Effect of Polybrominated Diphenylether (PBDE) and PCB on the Development of Reproductive Organs and Estrogen-Regulated Gene Expression in the Rat

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Summary

Polybrominated diphenylethers (PBDEs) are widely used as additive flame retardants in many different plastic materials and textiles at concentrations ranging from 5% to 30%. As they are not covalently bound, but admixed to polymers, they can be released into the environment, where they bioaccumulate as a result of their lipophilic characteristics. PBDEs have been detected in various biotic samples such as birds, eels, seals, whales, and also in human blood, adipose tissue, and breast milk. The relevance of PBDEs as environmental contaminants has been realized a few years ago, when it was demonstrated that concentrations in Swedish human milk have markedly increased over the last two decades.

Knowledge on PBDE toxicity is quite limited. The most sensitive end points of PBDEs *in vivo* so far known are effects on thyroid function, observed as induction of thyroid hyperplasia and alteration of thyroid hormone production in rats and mice; PBDEs were also found to have neurotoxic effects. Major data gaps include their effects on development, reproduction, and carcinogenicity.

The aim of our study was to provide information on the effects of PBDEs on development of a mammalian species, the rat, with special emphasis on reproductive organs. The basic hypothesis was that PBDEs, which bear some resemblance to PCBs, might interfere with the endocrine system, in the manner of an endocrine disruptor.

The congener selected was PBDE 99, (2,2',4.4',5- pentabromo-diphenylether), one of the congeners found in the biosphere, and present in human milk. In preliminary experiments, PBDE 99 had been found to be neurotoxic in newborn mice.

PBDE 99 was injected subcutaneosly to time-pregnant Long Evans rats from gestational day 10 (GD 10) to GD 18, at doses of 1 mg/kg and 10 mg/kg (9 injections). Additional groups of time-pregnant dams were treated with Aroclor 1254, an industrial PCB mixture, (10 mg/kg and 30 mg/kg), or with vehicle (olive oil). Aroclor 1254 was used as positive control, with the aim to compare PBDE effects with those of PCBs, which are well documented.

The rat offspring (F1 generation) were monitored from birth to adulthood. Survival rate and body weight were not affected by PBDE 99 indicating absence of general toxicity at the doses used. The onset of puberty was delayed in females (vaginal opening) and advanced in males (preputial separation). Data from organ weights of adult offspring indicate changes in ventral and dorsal prostate (1 and 10 mg/kg PBDE 99, respectively),

epididymis (both doses) and in ovaries (10 mg/kg PBDE 99). Aroclor 1254 delayed the onset of puberty in females at 30 mg/kg, anda affected the weight of epididymis, seminal vesicles, uterus, and ovaries at the dose of 10mg/kg. Animals exposed to 30 mg/kg of Aroclor 1254 exhibited a marked reduction of early postnatal survival rate and rarely reached adulthood. Females appeared to be more sensitive.

In order to obtain information on possible longterm effects at the molecular level, mRNA levels of the estrogen target genes insulin-like growth factor-I (IGF-I), estrogen receptor α (ER α) and ERβ, progesterone receptor (PR), and androgen receptor (AR) was performed in dorsal and ventral prostate and in uterus of adult offspring. mRNA levels were quantified by Real Time-PCR, with cyclophilin as reference.

In ventral prostate of adult offspring, PBDE 99 given at 1 mg/kg significantly down-regulated IGF-I, AR, and ERβ mRNA levels; the higher dose (10 mg/kg) down-regulated all the genes studied. Aroclor 1254 decreased IGF-I, AR, and ERβ mRNA levels at a dose of 10 mg/kg.

In dorsal prostate, prenatal administration of PBDE 99 resulted in a different effect spectrum. Adult offspring exhibited increased levels of AR mRNA (both PBDE 99 doses) and ERα mRNA (10 mg/kg), but reduced ERβ mRNA (both doses), while the level of IGF-I mRNA was unchanged. Aroclor 1254 increased IGF-I mRNA levels.

In uterus of adult offspring, developmental exposure to the low dose of PBDE 99 resulted in down-regulation of PR mRNA and up-regulation of ERβ mRNA. Exposure to the higher dose decreased PR and ERβ mRNAs. Aroclor 1254 decreased ERβ mRNA levels.

Additional experiments were conducted in order to assess the responsiveness of estrogen-regulated genes to estrogen. Adult male and female offspring exposed to PBDE 99 during development were gonadectomized in order to reduce and stabilize endogenous sex hormone levels, and injected with estradiol subcutaneously 2 weeks later. Animals were investigated 6 hours after estradiol injection.

The analysis of acute changes in mRNA levels (6 hours) revealed sex- and tissue-specific alterations in the sensitivity of estrogen-regulated genes to estradiol in adult offspring following exposure to PBDE 99 during development. The acute induction of IGF-I mRNA by estradiol was reduced in ventral prostate and uterus. Uterus further exhibited enhanced responses of PR mRNA (induction) and ERβ mRNA (suppression). These data indicate changes in the regulation of the respective genes. The changes in steady state mRNA levels and in response to estradiol could have resulted from actions of PBDE 99 during early ontogeny or at later stages, or from a combination of both.

In conclusion our results indicate that prenatal PBDE 99 exposure can affect the development of the rat gonadal axis, with changes in reproductive organ weights and in estrogen target gene expression. The action corresponds to effects of endocrine disruptors. The effect patterns differ in part from those of the PCB mixture, Aroclor 1254, indicating that the two groups of chemicals deserve separate consideration.

Zusammenfassung

Polybromierte Diphenyläther (PBDE) finden breite Anwendung als Flammschutzmittel in Plastikwaren und Textilien in Konzentrationen von 5 bis 30%. Da sie nicht kovalent gebunden, sondern dem Polymer zugemischt werden, können sie relativ leicht aus dem Material herausdiffundieren und in die Umwelt gelangen, wo sie dank ihren lipophilen Eigenschaften bioakkumulieren. PBDE-Kongenere wurden in verschiedensten biotischen Proben gefunden; z.B. in Vögeln, Aalen, Robben, Walen und in menschlichem Blut, Fettgewebe und Humanmilch. Die Relevanz der PBDE als Umweltkontaminantien wurde erst vor einigen Jahren erkannt, als bekannt wurde, dass die Konzentrationen in Humanmilch schwedischer Frauen in den letzten zwei Jahrzehnten exponentiell gestiegen sind.

Über die Toxizität der PBDE ist wenig bekannt. PBDE können *in vivo* die Schilddrüsenfunktion beeinflussen, es wurden Schilddrüsenhyperplasie und Veränderung der Schilddrüsenhormonkonzentrationen bei Ratten und Mäusen beobachtet. PBDE können auch neurotoxisch wirken. Was fehlt, sind Daten über Entwicklung, Reproduktion und Kanzerogenität.

Das Ziel unserer Untersuchungen war, mehr über mögliche Wirkungen von PBDE auf die Entwicklung einer Säugetierspezies, der Ratte, speziell über die Entwicklung der Reproduktionsorgane, zu erfahren. Die Hypothese war, dass PBDE, die Ähnlichkeiten mit PCB besitzen, möglicherweise wie ein endokriner Disruptor mit der Entwicklung endokriner Systeme interagieren könnten.

Für die Untersuchungen wurde das Kongener PBDE 99, (2,2',4.4',5-Pentabromo-diphenyläther) ausgewählt. PBDE 99 wurde in der Biosphäre, z.B. in Humanmilch, nachgewiesen, und hatte sich in präliminären Experimenten an neugeborenen Mäusen als neurotoxisch erwiesen. PBDE 99 wurde trächtigen Long Evans Ratten mit zeitlich kontrollierter Begattung in Dosierungen von 1 mg/kg und 10 mg/kg subkutan injiziert, und zwar vom Schwangerschaftstag 10 bis 18 (9 Injektionen). Eine zusätzliche Gruppe trächtiger Rattenweibchen wurde in analoger Weise mit Aroclor 1254, einer industriellen PCB-Mischung (10 mg/kg oder 30 mg/kg), behandelt. Kontrollen erhielten das Vehikel (Olivenöl). Aroclor 1254 diente als Positivkontrolle, mit dem Ziel, PBDE-Effekte mit denjenigen von PCB zu vergleichen. Letztere sind sehr gut dokumentiert.

Die postnatale Entwicklung der Nachkommen (F1-Generation) wurde von Geburt bis ins erwachsene Alter beobachtet. Überlebensrate und Körpergewicht wurden durch PBDE 99

nicht beeinflusst, was anzeigt, dass PBDE 99 in den verwendeten Dosierungen keine allgemein toxischen Effekte ausübte. Der Beginn der Pubertät war bei weiblichen, PBDE 99-exponierten Jungtieren verzögert (Vaginalöffnung), bei Männchen vorverschoben (Ablösung des Präputiums). Änderungen des Organgewichts zeigten bei adulten Nachkommen ventrale und dorsale Prostata (1 bzw. 10 mg/kg PBDE 99), Epididymis (beide Dosisgruppen) und Ovarien (10 mg/kg PBDE 99). Aroclor 1254 verzögerte den Pubertätseintritt bei Weibchen (30 mg/kg) und beeinflusste die Organgewichte von Nebenhoden, Samenblasen, Uterus und Ovarien adulter Nachkommen in der Konzentration von 10 mg/kg. Jungtiere der Dosisgruppe 30 mg/kg Aroclor 1254 zeigten eine markante Reduktion der postnatalen Überlebensrate und erreichten kaum das Erwachsenenalter. Weibchen schienen empfindlicher zu sein.

Um Aufschluss über Langzeiteffekte auf molekularer Ebene zu gewinnen, wurden die mRNA-Konzentrationen der Estrogen-Zielgene Insulin-like Growth Factor-I (IGF-I), Estrogen-Rezeptor α (ERα) und ERβ, Progesteron-Rezeptor (PR) und Androgen-Rezeptor (AR) in der dorsalen und ventralen Prostata und im Uterus adulter Jungtiere mit Hilfe der Real Time PCR Methode bestimmt. Cyclophilin diente als Referenzgen.

In der ventralen Prostata adulter Jungtiere senkte PBDE 99 in einer Dosis von 1 mg/kg die mRNA-Konzentrationen von IGF-I, AR, und ERβ, während bei der höheren Dosis (10 mg/kg) alle untersuchten Gene herunterreguliert wurden. Aroclor 1254 (10 mg/kg) reduzierte IGF-1, AR, und ERβ mRNA. Die dorsale Prostata zeigte ein anderes Effektspektrum. Pränatale Verabreichung von PBDE 99 erhöhte bei adulten Jungtieren die Konzentrationen von AR mRNA (beide Dosisgruppen) und ERα mRNA (10 mg/kg), reduzierte ERβ mRNA (beide Dosierungen), hatte aber keine Wirkung auf die IGF-I mRNA Konzentration.

Im Uterus fand sich unter der niedrigen PBDE 99-Exposition eine Reduktion von PR mRNA und ein Anstieg von ERβ mRNA. Bei der höheren Dosis wurden die Konzentrationen von PR mRNA und ERβ mRNA herunterreguliert. Aroclor 1254 reduzierte die Konzentration von ERβ mRNA.

Zusätzliche Experimente wurden ausgeführt, um die Empfindlichkeit der Estrogen-regulierten Gene auf Estrogen zu untersuchen. Ratten beiderlei Geschlechts, die während der Entwicklung PBDE 99-exponiert waren, wurden im jung-adulten Alter gonadektomiert, zur Reduktion und Stabilisierung der Konzentrationen der endogenen Geschlechtshormone. Zwei Wochen später wurde den Tieren Estradiol subkutan injiziert. Sechs Stunden danach wurden sie untersucht.

Die Analyse zeigte geschlechts- und gewebeabhängige Veränderungen der akuten Reaktion Estrogen-regulierter Gene auf Estradiol in ventraler Prostata und Uterus. Die akute Induktion von IGF-1 mRNA durch Estradiol war in ventraler Prostata und Uterus herabgesetzt. Im Uterus fand sich ausserdem eine verstärkte Induktion von PR mRNA und eine verstärkte Suppression von ERβ mRNA. Dies spricht für eine veränderte Regulation dieser Gene. Die Änderungen der mRNA-Konzentrationen im stationären Zustand und die veränderte Sensitivität für E2 könnten durch den Einfluss von PBDE 99 auf die frühe Ontogenese und/oder durch Wirkungen in der späteren postnatalen Entwicklung verursacht worden sein.

Zusammenfassend zeigen unsere Resultate, dass PBDE 99-Exposition die Entwicklung des Reproduktionstraktes männlicher und weiblicher Ratten auf der Organebene und auf molekularer Ebene beeinflussen kann. Die Wirkung entspricht jener von endokrinen Disruptoren. Das Effektmuster von PBDE 99 weicht zum Teil von jenem des PCB-Gemisches, Aroclor 1254, ab, was eine getrennte Betrachtung der zwei Chemikaliengruppen nahelegt.

Contents

1. Introduction

1.1 General Introduction

During recent years, chemicals able to interfere with endocrine systems of humans and animals, have received increasing attention. So far most of the literature deals with xenoestrogens, chemicals that interfere with the female sexual hormone estrogen.

The effects of the xenoestrogens were first discovered in rivers and below influxes of sewage treatment plants, where an increased number of fish with defects in liver, kidneys and reproductive organs were observed (BUWAL, 1999). In addition males produced increased amounts of the female egg-yolk protein vitellogenin (Munkittrick et al., 1991; Sumpter and Jobling 1993). Synthesis of this protein is induced by estrogens or “estrogen like” substances in water.

In many animal species like marine mammals a decrease in fertility is seen (Colborn and Smolen, 1996). Highly lipophilic chemicals bio-accumulate in living organisms and are stored in adipose tissues like in the blubber of marine mammals. Such chemicals are supposed to contribute to reproductive dysfunction.

There is increasing evidence that humans can also be affected by exposure to contaminants in the food chain or by accidents. The high discharge of TCDD (Dioxin) in the Seveso accident in 1976 led to an altered sex ratio (decrease from 51% to 35% in births of boys). High TCDD contamination with serum concentrations of 15 to 20 ng ppt TCDD of the fathers at peripubertal age could be correlated with the changes in sex ratio of their children that normalized in 1985, when TCDD contamination had decreased (Mocarelli et al., 2000).

Another highly discussed topic is the earlier onset of puberty in girls. It is known from animal studies, that exposure to xenoestrogens can reduce the age of onset of puberty in females, while exposure to compounds that interfere with androgens delay puberty in males (Howdeshell et al., 1999; Gray et al., 1999).

Chemicals, such as diethylstilbestrol (DES), bisphenol A (BPA), and mixtures of polychlorinated biphenyls (PCB) (Eroschenco et al., Katsuda et al., 2000, Vreugdenhil et al., 2002) may have long-term effects on human health due to their estrogenic activity. After fetal exposure, DES and BPA increased androgen receptor (AR) binding activity of the prostate in adult rats at low doses (from 100 ng/kg/day to 50µg/kg/day) and altered

prostate and epididymis size (vom Saal et al., 1998; Atanassova et al., 2000; Declos et al., 2001; Stoker et al., 1999).

From this point of view, it is evident that there is a need to investigate more closely the effects of several compounds on humans and animals.

1.2 The Flame Retardants

The idea of flame retardant material dates back to about 450 BC, when the Egyptians used alum to reduce the flammability of wood. The Romans (about 200 BC) used a mixture of alum and vinegar to reduce the combustibility of wood. Today, there are more than 175 chemicals classified as flame retardants. The four major groups are inorganic, halogenated organic, organophosphorus and nitrogen-based flame retardants.

Advances in polymer science over the past 50 years has led to the introduction of a large number of polymers with different properties and applications. As a result, we are surrounded by a wide variety of polymers in clothing and furniture, vehicles and computers. In fact modern cars contain in excess of 100 kg of various polymers. Most of these polymers are petroleum-based and hence are flammable. In order to meet fire safety regulations, flame retardants are applied to combustible materials such as plastics, wood, paper, and textiles. Flame retardants are materials added or applied to a material to increase the fire resistance of that product. With the increasing usage of polymeric materials in construction, electronic and computer equipment, global market demand continues to grow substantially (Alae et al., 2003).

The Brominated Flame Retardants

Brominated flame retardants (BFRs) are used in a variety of consumer products and several of those are produced in large quantities. They are produced via direct bromination of organic molecules or via addition of bromine or alkenes.

BFRs are divided into three subgroups depending on the mode of incorporation of these compounds into the polymers: brominated monomers, reactive and additive. A brominated monomer such as brominated styrene or brominated butadiene is used in the production of brominated polymers, which are then blended or introduced into the feed prior to polymerisation, resulting in a polymer containing both brominated and non-brominated

monomers. Reactive flame retardants, such as tetrabromobisphenol A, are chemically bonded into the plastics. Additive flame retardants, which include Polybrominated diphenyl ethers (PBDEs) are simply blended with polymers, and are more likely to leach out of the products (Hutzinger et al., 1987).

Polybrominated Diphenylethers

Polybrominated diphenyl ethers (PBDEs) are additive flame retardants and the next highest production group of BFRs currently in use. They are used in plastic (concentration 5-30%) and textile coatings. The general chemical formula is C12H(9-0)Br(1-10)O with the sum of H and Br atoms always equal to 10. They are molecules containing 10 hydrogen atoms, any of which can be exchanged with bromine, resulting in 209 possible congeners and divided into 10 congener groups. The structure of PBDEs is similar to that of PCBs, hence its nomenclature is also used for PBDEs.

PBDEs are produced at three different degrees of bromination, i.e. Penta-BDE, Octa-BDE and Deca-BDE and classified according to their average bromine content. Commercial PBDEs are quite resistant to physical, chemical, and biological degradation. The boiling point of PBDEs is between 310 and 425°C and their vapor pressure is low at room temperature. PBDEs are lipophilic, and their solubility in water is low, especially for the higher brominated compounds. The global production is about 40,000 tons per year (Darnerud et al., 2001).

The Penta-brominated Diphenylethers

The two major congeners present in the commercial Penta-BDE are: 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and 2,2',4,4',5-pentabromodiphenyl ether (BDE-99). The formation of these two congeners is due to the presence of other precursors, the congener mostly favored is BDE-47 and its continued bromination yields mainly the BDE-99 and to a lesser extent 2,2',4,4',6-penta-BDE (BDE-100). The percentages of BDE-99 and BDE-100 in commercial mixtures are 35% and 6.8%, respectively. The favored formation of BDE-99 might result from the *ortho/para* directing property of the bromine atoms in BDE-47 and/or steric hindrance.

Penta-BDE formulation is a viscous liquid, which contains 70% bromine by weight, consists of 41-42% tetra-BDEs (mainly BDE-47), 44-45% Penta-BDEs (predominantly

BDE-99 and to a lesser extent BDE-100). The formulation of Penta-BDE contains the three predominant congeners (BDE-47, BDE-99 and BDE-100), which are also found in biological matrices including human tissue (de Wit, 2002).

Figure 1.1: The Penta-brominated diphenyl ethers general formula.

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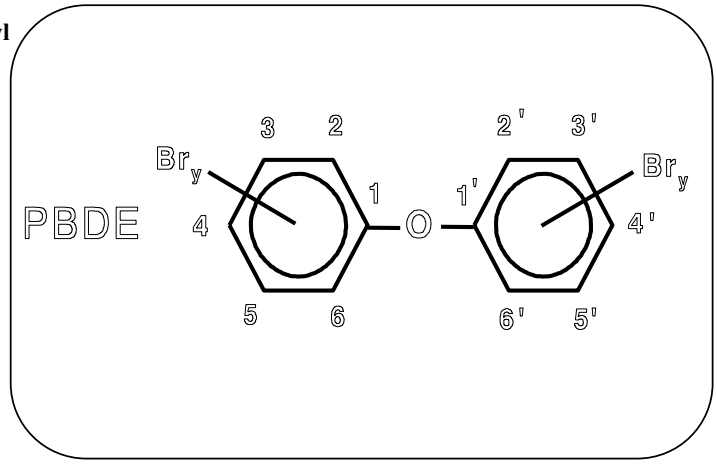


Because of the widespread production and use of PBDEs, their high affinity to particles, and their lipophilic characteristics, several PBDE congeners bio-concentrate and bio-accumulate in the environment in a manner similar to the structurally related polychlorinated biphenyls (PCBs) (Pijnenburg AMCM, et al., 1995).

PBDEs have been detected in various biotic samples such as birds, seals, whales, and even in human blood, adipose tissue, and breast milk (de Boer J. et al.,1998; Stanley JS. et al., 1991).

The relevance of PBDEs as environmental contaminants has been demonstrated by their accumulation in human breast milk, where concentrations in Swedish women have increased over the last 2 decades from 0.07 ng/g lipid weight in 1972 to 4.02 ng/g lipid weight in 1998 (fig. 1.2) (Meroyntè D. et al.,1999).

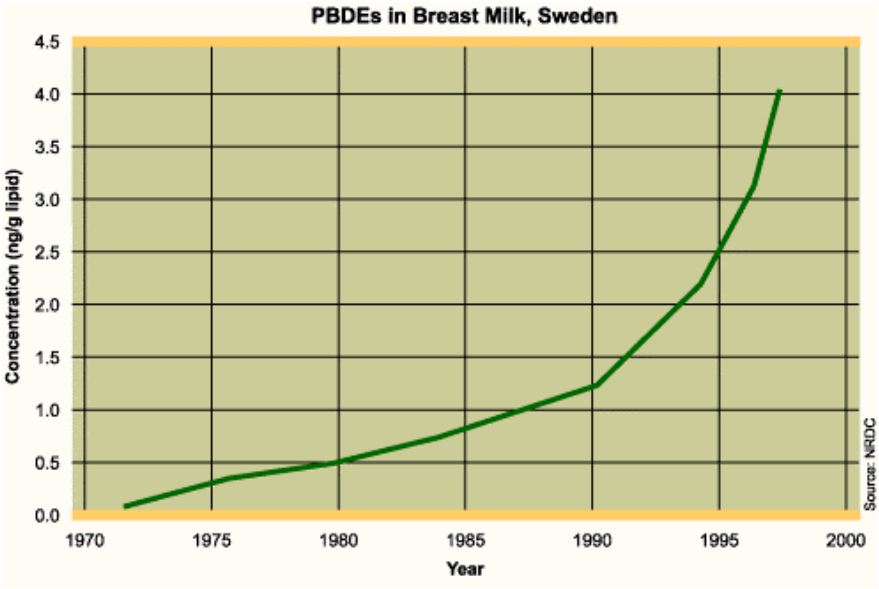


Figure 1.2: PBDEs increase in Swedish human breast milk over almost 2 decades: from 1972 to 1998 (Meynrote, 1999).

Although PCB concentrations in wildlife are still higher than PBDE concentrations, the former are declining over the same time period, while PBDE levels are rising.

The most sensitive end points of PBDE toxicity *in vivo* so far include effects on thyroid function, observed as induction of thyroid hyperplasia and alteration of thyroid hormone production (i.e., lowering of free and total thyroxine (T4) concentrations) in rats and mice (Darnerud PO. Et al., 1996; Fowels JR. et al., 1996).

Consistent with these findings is the recent observation that several pure PBDE congeners were able to displace T4 from transthyretin (TTR; a plasma transport protein of thyroid hormones) *in vitro,* after metabolic conversion to hitherto unidentified metabolites (Meerts et al., 2000). These phenomena have also been observed for

other organhalogen compounds with similar structure: the PCBs and their hydroxylated metabolites (Brucker-Davis 1998).

Another property that PBDEs share with PCBs and the polybrominated biphenyls (PBBs) is the dioxin-like, Ah receptor-mediated induction of cytochrome P450 1A1 and 1A2 *in vitro* (Brouwer A. et al., 1998) and *in vivo* (Hamberg A. et al., 1998).

Some studies have indicated that hydroxylated PBDEs are of potential environmental importance. In liver microsomes of rats, several PBDE congeners were bio-transformed to metabolites (Meerts et al., 2000).

PBDEs: International and National Legislation and Restrictions

The need for restrictions on certain PBDEs in different types of plastics and textiles is currently being discussed within the EU. Consequently, the use of certain brominated flame retardants (PBB and tris(2,3-dibromopropyl)phosphate) in textiles has already been banned. A document of the Organization for Economic Co-operation and Development (OECD) document proposes precautions for each type of PBDEs. These include recommendations to stop the use of certain compounds (mainly tetra- and penta-BDEs), as well as to limit occupational exposure (OECD, 1994). Also the Paris Commission for the Prevention of Marine Pollution is working toward restricting and phasing out PBDEs and PBBs.

An example of a national restriction is the Swedish government's intention to ban PDEs and PBBs in products sold on the Swedish market (The Swedish National Chemicals Inspectorate, 1999)

1.3. PCBs

Polychlorinated biphenyls (PCBs) consist of 12 carbon atoms, forming two aromatic phenyl rings attached to one another through a carbon-carbon bridge, and 10 atoms that can be either hydrogens or chlorines (fig. 3).

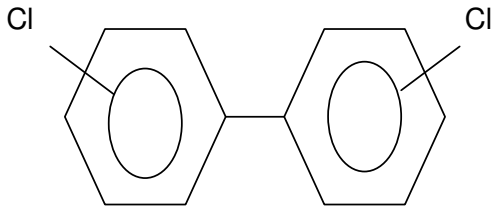


Figure 1.3: Structure of PCB.

Chlorine increases the stability and decreases flammability of these compounds.

All PCBs are lipophilic and pratically insoluble in water. Lipophilicity increases by increasing rate of chlorination. Their boiling point varies from 300 to 400°C. They resist high temperatures and oxidising conditions.

Their electrical conductivity is very low which made them suitable cooling liquids for electrical equipment and they have been used as insulating materials in electrical

equipment, as plasticizers and for other industrial purposes (lubricants, cutting oils, adhesives and etc.).

They were manufactured from 1930 to 1970s or 1980s (varying in different countries), and the total production was in excess of a million tonnes. They have spread to the environment in accidents (such as transformer fires or leaks), from volatilisation of waste landfills and incineration of mixed municipal waste. The major source of human exposure was food, expecially fatty food.

Effects on humans were observed as liver enlargement and immunological problems. (Fowels et al., 1994).

Trade names for PCBs include the following: Apirolio, Aroclor, Clophen, Fenchlor, Kanechlor, Phenochlor, Pyralene, Pyranol, Pyroclor, Santotherm Fr, and Sovol. Sometimes the trade name indicates the degree of chlorination, e.g. Aroclor 1254 contains 54% of chlorine, 12 indicates the number of carbon atoms.

1.4 Endocrine Disruptors

An endocrine disruptor (EDC) is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently can cause adverse health effects in an intact organism, or its progeny, or in (sub)populations.

The last decades have witnessed growing scientific concerns and public debate over the potential adverse effects that may result from exposure to a group of chemicals that have the potential to alter the normal functioning of the endocrine system in wildlife and humans.

Concerns regarding exposure to these EDCs are due primarily to:

1. Adverse effects observed in certain wildlife, fish, and ecosystems.
2. The increased incidence of certain endocrine-related human diseases.
3. Endocrine disruption resulting from exposure to certain environmental chemicals observed in laboratory animals.

These concerns have stimulated many national governments, international organizations, scientific societies, the chemical industry, and public interest groups to establish research programs.

Endocrine Mechanisms of Action

Research has clearly shown that EDCs can act at multiple sites via multiple mechanisms of action. Receptor-mediated mechanisms have received the most attention, but other mechanisms (e.g., hormone synthesis, transport, and metabolism) have been shown to be equally important. For most associations reported between exposure to EDCs and a variety of biologic outcomes, the mechanism(s) of action are poorly understood. This makes it difficult to distinguish between direct and indirect effects and primary versus secondary effects of exposure to EDCs.

It also indicates that considerable caution is necessary in extrapolating from *in vitro* data to *in vivo* effects, in predicting effects from limited *in vivo* data, and in extrapolating from experimental data to the human situation.

Despite an overall lack of knowledge of mechanisms of action of EDCs, there are several examples where the mechanism of action is clearly related to direct perturbations of endocrine function and ultimately to adverse *in vivo* effects.

Important aspects to be considered are:

- Exposure to EDCs during the period when "programming" of endocrine system is in progress may result in a permanent change of function or sensitivity to stimulation/inhibitory signals.
- Exposure in adulthood may be compensated for by normal homeostatic mechanisms and may therefore not result in any significant or detectable effects.
- Exposure to the same level of an endocrine signal during different seasons may produce different effects.
- Because of cross talk between different components of the endocrine systems, effects may occur unpredictably in endocrine target tissues other than the system predicted to be affected.

Today there is little knowledge of the relationship between molecular events and potential adverse health outcomes.

Dose-Response Relationships

The issue of dose-response relationships is perhaps the most controversial issue regarding EDCs. One of the reasons is that EDCs often act by mimicking or antagonizing the action of naturally occurring hormones. These hormones (often more potent than exogenous EDCs) are present at physiologically functional concentrations, so the dose-response considerations for EDCs are often different from other environmental chemicals, which are not acting directly on the endocrine system. Reports of low-dose effects of EDCs are highly controversial and the subject of intense research. The existence of low-dose effects has been confirmed by an international panel (Endocrine Disruptors Low Dose Peer Review, 2001). Dose-response relationships are likely to vary for different chemicals and endocrine mechanisms. Timing of exposure is absolutely critical to the understanding of dose-response relationships for EDCs. This is true for both wildlife and humans and for cancer as well for developmental, reproductive, immunological, and neurobiological effects.

Effects in Wildlife

Several field and laboratory studies have shown that exposure to certain EDCs has adverse effects in some wildlife species and populations. These effects vary from subtle changes in the physiology and sexual behaviour of species to permanently altered sexual differentiation. Most of the data originate from Europe and North America. Aquatic species (at the top of food chain) have been most frequently studied, but effects have also been observed in terrestrial species. Some adverse effects observed in certain species are likely to be endocrine mediated, but in most cases, the casual link between exposure and endocrine disruption is unclear. Examples of cases observed:

- ✓ Mammals: exposure to organochlorines (PCBs, DDE) has been shown to adversely impact the reproductive and immune function in Baltic seals, resulting in marked population declines (Reijnder PJH, 1980; 1986; and 1990).
- ✓ Birds: eggshell thinning and altered gonadal development have been observed in birds of prey exposed to DDT, resulting in severe population declines. A syndrome of embryonic abnormalities has been observed in fish-eating birds and can be directly related to PCB exposure (Fry DM et al., 1987; Gilbertson M. et al., 1991; NcArthur MLB et al.,1983).
- ✓ Reptiles: a pesticide spill in Lake Apopka provides a well-documented example of potential EDC effects on population decline in alligators. Several hypotheses have been proposed to explain that organochlorine contaminants induced endocrine disruption (Guillette LJ et al., 1994 and 1999a).
- ✓ Amphibians: population declines has been observed in both pristine and polluted habitats worldwide (Vos JG et al., 2000).
- ✓ Fish: there is extensive evidence that chemical constituents present in pulp and paper mill effluent and sewage treatment effluents can affect reproductive endocrine function and contribute to alteration in reproductive development (Kime et al., 1999; Mellanen et al., 1999).

Human Health Effects

It is difficult to compare and integrate results from diverse human studies, because data are often collected at different time periods, using different experimental designs and at different exposure conditions (Farrow S., 1994). Often exposure data are completely lacking. Of particular concern is the lack of exposure data during critical periods of development that influence later functioning in adult life (Berman NG., 1996). Furthermore, the concentrations and potencies of endogenous hormones and phytoestrogens are generally higher than those of exogenous chemicals, but the situation changes with the life cycle. For example, endogenous estrogen-concentrations are very low in embryos and fetuses. Also, the kinetics (transport, tissue distribution, metabolism) differ greatly between endogenous hormones, phytoestrogens and industrial chemicals. Despite these difficulties in interpretation, exposure to EDCs has been suggested to play a role in adverse health outcomes, and concerns remain.

Examples illustrating these concerns:

- Reproductive effects: a number of studies have reported a decline in human sperm quality in several countries, but there are no firm data that directly addressed the possible cause and effect relationship between declining sperm quality and exposure to EDCs (Nelson et al., 1974; Irvine et al., 1996) mainly because of the lack of data on pre- and postnatal exposure levels. Declining sex ratios (fewer males) have been recorded in a number of regions and countries. There is also evidence that unidentified external influences are associated with such changes, but the mechanisms are unknown (Bush B. et al., 1986). This is supported by the data of the Seveso accident mentioned above (Mocarelli et al., 2000).
- Endometriosis: exposure to certain EDCs has been reported to be associated with endometriosis but, the studies remain equivocal (Guarnaccia & Olive, 1998; Boyd et al., 1995).
- Neural function: alteration in sexually dimorphic play behaviour have been linked to prenatal PCB exposure (Vrengdemhil et al., 2002).
- Immune function: exposure to environmental chemicals has shown to alter immune function in humans and animals. However, it is not clear whether such

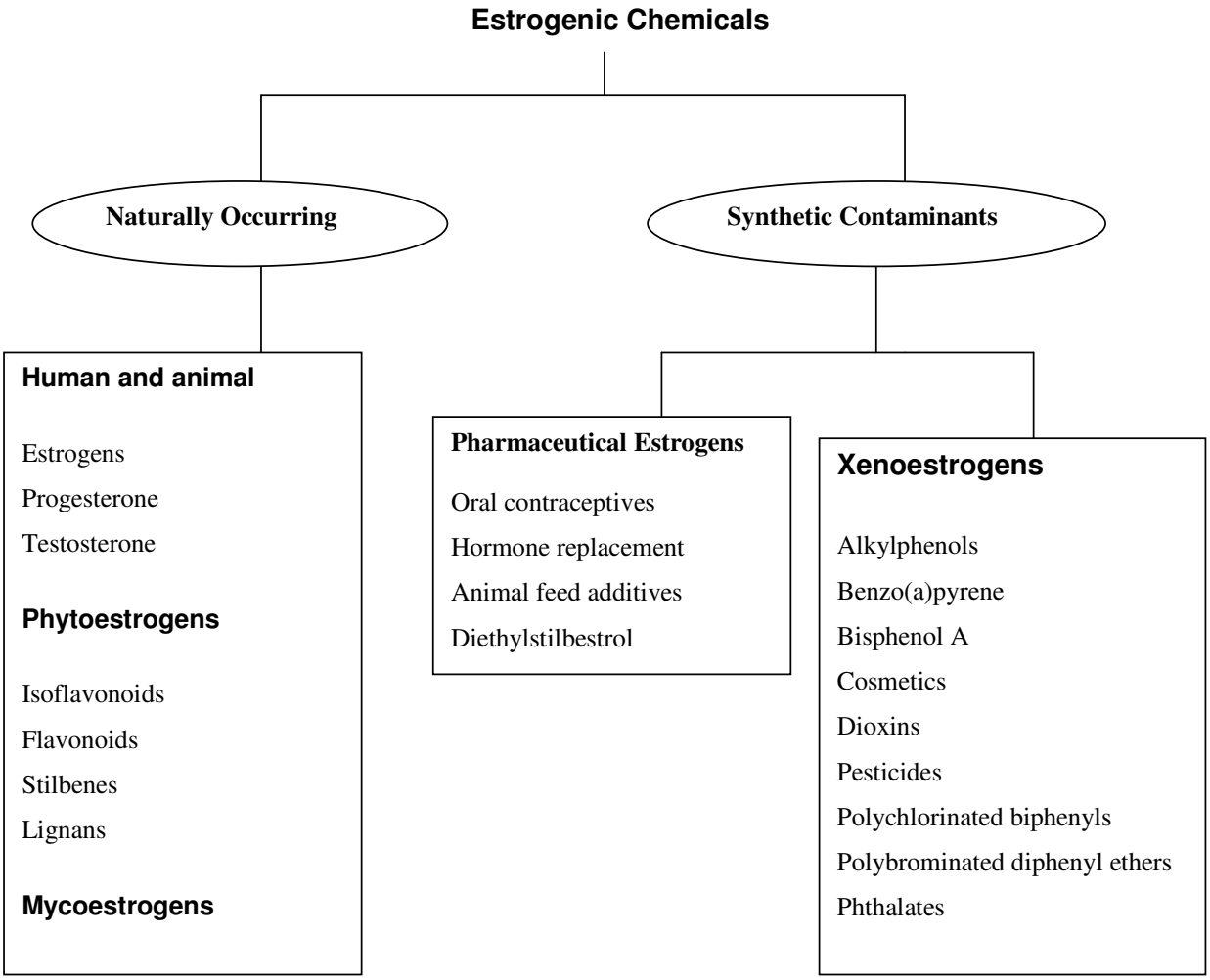
impaired function is due to endocrine-mediated mechanisms (Chen RC. Et al., 1985).

- Cancer: increases in the incidence of certain cancers in hormonally sensitive tissues with time are often cited as being possibly related to EDCs because of the enormous increase in production volume of chemicals since the early 20th century (Russo & Russo, 1996).
- Breast cancer: numerous epidemiological and experimental laboratory studies have been conducted to determine whether environmental EDCs may contribute to an increased risk of breast cancer, but the current scientific evidence remains uncertain about exposure to environmental EDCs and increased risk of breast cancer (Stanford et al., 1995; Pike MC et al., 1993; Adami et al., 1995).
- Prostate cancer: exposure to certain pesticides and organochlorines has been linked to increases in the incidence of prostate cancer in a few limited studies (Bertazzi et al., 1987; Brown DP, 1987; Morrison et al., 1993).
- Thyroid cancer: a direct association between exposure to EDCs and thyroid cancer has not been demonstrated (Porterfield & Hendry, 1998).

Overall, the biological plausibility of possible damage to certain human functions, reproductive and developing systems, from exposure to EDCs seems strong when viewed against the background of known influences of endogenous and exogenous hormones on many of these processes. Furthermore, the evidence of adverse outcomes in wildlife and laboratory animals exposed to EDCs substantiates human concerns.

The age at which the exposure occurs in humans is also critical to assessing potential effects of EDCs. *In utero*, neonatal, childhood, and puberty appear to be critical developmental periods potentially susceptible to interference by EDCs. For example, it has been demonstrated that the effects of exposure to PCBs on neurobehavioral function in children will vary depending upon whether exposure occurs prenatally or postanatally (Jakobson and Jakobson 2001).

The following scheme presents groups of substance able to perform endocrine disruption:



Sources

Some environmental EDCs are released into the environment intentionally (e.g. pesticides), but for most of the environmental contaminants, release is unintentional. It could occur throughout part or all of the chemical's life cycle (e.g. manufacturing, use, disposal). "Dioxin-like" chemicals are formed intentionally as by-products in a variety of industrial and combustion process (Fara 1999; Toblin 1986). Leakage from landfill areas and distribution via sewage sludge are also sources of exposure (Daughton et 1999).

Exposure to naturally occurring EDCs such as phyto- and fungal estrogens, which are important components of some human and wildlife diets, occurs globally. The isoflavonoid phytoestrogens are found in soy and legumes, the lignanes in grains and many fruits and vegetables. All have relatively short-half lives in humans, and metabolites can be detected in urine and feces. Phytoestrogens have also been detected in effluents from pulp mills, resulting in reproductive effects in certain fish species.

Exposure can occur via air, water, soil, sediment, and food and consumer products. The chemical may then enter the organism by ingestion, inhalation, or skin contact across cell membranes and then be absorbed into the bloodstream (Crosby, 1998).

Some chemicals considered as EDC and their effects

PCBs. The manufacturing of PCBs has been banned in many countries since 1970a (de March, 1998). However, because of their long lifetime in old electrical equipment, continued use in some parts of the world, and their persistence and bioaccumulation, populations continue to be exposed.

DDT (Dichlorodiphenyl tricthlroethane). The manufacturing and use of a number of pesticides, including DDT, have been banned or greatly restricted. However DDT is still used in developing countries. While the concentration of DDT metabolites is decreasing in northern hemisphere population, in southern hemisphere these compounds are still present at relatively high concentrations (Torres-Arreola, 1999). During the 1960s and 1970s, when DDT was present in the North American environment at greater concentrations, population of several sensitive bird species declined because of unsuccessful incubation of eggs due to abnormally thin egg shells (Cooke, 1973)

Phthalates. Phthalates are diester derivates of phthalic acid used primarily as plasticizers to make plastic products more flexible. The main property of these plasticizers is to not become a permanent (chemically bonded) part of the plastic matrix during manufacturing process. They can migrate from plastic to environmental matrices under certain conditions. As a result they become ubiquitous in our environment (Steiner, 1999). Some phthalates induce antiandrogenic effects in fetal males. Male rat pups exposed during sexual differentiation exhibited malformations in androgen-dependent tissues (Gray, 1990-2000)

Atrazine. Atrazine is a member of the triazine herbicide family and has been widely used for weed control in agricultural crops. It is frequently found in surface water and groundwater. Because it may be found in drinking water, its use has been banned or

restricted in many countries. In the context of EDCs, there is concern about atrazine related to the development of mammary tumors in exposed rats (Elridge, 1994; Stevens, 1994).

1.5 The nuclear receptor family

The nuclear receptor family consists of proteins that mediate the actions of many important cell regulators. These include the steroid hormones (estrogens, progestins, androgens, glucocorticoids, mineralocorticoids, ecdysteroids, vitamin D), thyroid hormones, and retinoids. In contrast to receptors for peptide hormones, which are located in the cell membrane and evoke a second messenger to deliver the regulatory signal, the nuclear receptors are present within the cell. After associating with their respective ligands, they eventually act as transcription factors in the cell nucleus to enhance the expression of specific genes. Agents that act by way of nuclear receptors influence the behaviour of many tissues in vertebrate species. Often certain cells are especially responsive. For example, the gonadal steroid hormones control growth, differentiation, and function of reproductive and accessory sex tissues: estrogens and progestins in the female (uterus, vagina, oviduct, mammary gland) and androgens in the male (prostate, and seminal vesicles). They also influence many other tissues, including skin, hair, bone, pituitary, and the central nervous system. Steroid hormone receptors are posphorylated proteins containing sulfhydryl groups necessary for ligand interaction. In their native state, steroid receptors are complex entities, consisting of a hormone-binding protein associated with other macromolecules.

The important function of the steroid is to convert the native receptor to a biochemically functional state that can bind to the DNA and enhance gene transcription (Gorski et al., 1968).

Many studies demonstrated that hormone induced transformation of the native receptor to a form that can interact with target genes and that, is a general phenomenon for all classes of steroid hormones, but not for thyroid hormones (Jensen, 1990). Much of the native receptor for steroid hormones are present in the nucleus. To explain steroid hormone action, the “two-step” theory was introduced, which takes its origin from the mechanism proposed for the estradiol-receptor complex. Following this mechanism, there is a cytoplasmic-nuclear equilibrium of the receptor and the cytoplasmic level is continually

replenished from a nuclear pool, on the other hand there are receptors located only in the nucleus. Once the hormone arrives in the nucleus, receptor transformation and genome binding are initiated (Jensen, 1991).

Mechanisms proposed:

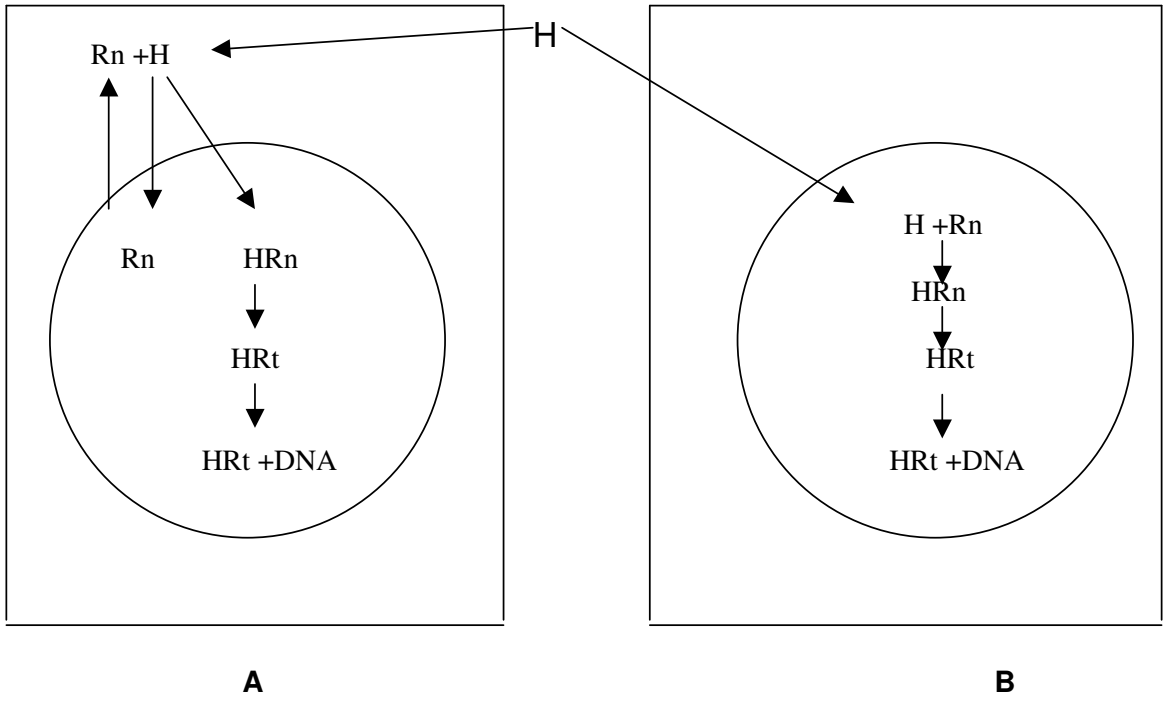


Figure 3: Schematic representation of hormone-receptor interaction in target cell. H, hormone; Rn native (untrasformed) receptor; Rt, transformed receptor. (A) Extranuclear receptor in equilibrium with nuclear pool of loosely bound native receptor. (B) Native receptor confined to the nucleus (Jensen).

Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is a cell protein that initiates many of the effects of dioxin-like chemicals. Its primary function in the body is uncertain, and it is structurally related to other important cell proteins involved for instance in rhythmic functions (clock proteins) and organ development.

When a dioxin compound enters the cell, it binds to AhR, this moves from cellular cytoplasm to nucleus, forms an heterodimer with another protein, ARNT (Ah Receptor Nuclear Translocator), and this heterodimer binds to DNA. This binding initiates the activation of a number of genes depending on the binding site of the dimer.

The environmental toxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is a high affinity ligand for the AhR and has extensively been used to investigate AhR-mediated biochemical and toxic responses. TCDD modulates several endocrine pathways including inhibition of 17β-estradiol-induced responses in the immature and ovariectomized rodent uterus and mammary gland and in human breast cancer cell lines (Safe S, et al., 2000).
Certain PBDE congeners can act as AhR agonists or antagonists (Meerts et al., 1998a). Pyrolysis and photolysis of PBDEs can lead to formation of other hazardous compounds such as Polybrominated dibenzo-*p*-dioxins and polybrominated dibenzofurans (Buser, 1986; Watanabe et al., 1987; Dumbler et al., 1989; Bruckmann & Hackhe 1989).

Steroid Receptors

A major mode of action of steroid hormones is regulating gene activity though nuclear receptors, which represent ligand-activated transcription factors that share common structural features (Kumar, 1999; White, 1998).
To this end, the receptor proteins possess characteristic domains corresponding to their specific functional roles, for example, for DNA binding (DBD) and ligand binding (LBD), two transcription activation domains (ligand-dependent, AF2, and ligand-independent, AF1), sequences for nuclear localization (NLS), for dimerization, and for interaction with nuclear and cytoplasmic proteins.
The ligand-activated receptor, as dimer, interacts with specific DNA binding sites (hormone response elements, HRE), and with a series of nuclear proteins, among them coactivators, acting positively on transcription, and corepressors, having negative effects on RNA synthesis (Motsatsou, 2003).
Nuclear receptors can also act as monomers, not by direct binding to DNA, but indirectly, through interaction with other DNA binding regulatory proteins (Reichardt, 1998). Furthermore, nuclear receptors can cross-talk with other regulatory agents, such as growth factors, cytokines, and neurotransmitters, in the absence of cognate ligand, by way of phosphorylation of the nuclear receptors at serine/threonine residues by phosphokinases activated by these molecules (Knazir, 1990).

1.6 Estradiol

17 β-Estradiol (E2) is a versatile hormone that acts on the uterus to stimulate cell proliferation and to increase sensitivity of the uterus to progesterone. New knowledge comes from ER knockout mouse models (αERKO and βERKO). Based on these, it appears that ERα plays the predominant role in mediating the effects of E2 in the uterus.

Estradiol exerts its effects in target organs by binding to ERs and modulating the expression of various proteins such as growth factors. In the uterus one of such factor is insulin-like growth factor I (IGF-I). It was shown (Klotz, 2000) that E2 administartion to ovariectomized adult female mice and rats results in an increase in uterine IGF-1 transcripts (klotz, 2000).

1.7 Estrogen Target Genes

ANDROGEN RECEPTOR

The development, maintenance and functions of the prostate gland requires the activity of androgen receptors (AR).

AR is a steroid hormone receptor that when activated directs the expression of target genes by acting as a DNA-binding transcription factor. AR interacts with specific DNA sequences or with other proteins in the regulatory regions of target genes, and recruits cofactors that either enhance or inhibit the rate of transcription, depending on the nature of the regulatory element. Intracellular signalling pathways, cell cycle proteins, and other factors can modulate AR by modifying the receptor or interacting with it physically, providing a means for cross-talk between hormonal and growth factor signals.

AR is expressed in the secretory epithelial cells, the surrounding periacinar stromal cells, and in perivascular smooth muscle of the prostate.

Few direct targets of AR in the prostate are known, and the genes immediately downstream of AR in the prostate that regulate proliferation and differentiation remain largely uncharacterised. Because AR acts primarily as transcription factor, differential

gene expression studies are an ideal means to study the effects of AR in prostate cells (Pascale, 2003).

ESTROGEN RECEPTORS

Estrogen regulates the growth and differentiation of the uterus via binding to estrogen receptors (ERs), members of the nuclear receptor family of transcription factors. The diverse effects of estrogen on a wide range of target tissues are mediated by estrogen receptors (ERs), nuclear transcription factors that are activated either upon binding of estrogen and estrogen-like ligands or via cross-talk, most likely through phosphorylation of the unliganded receptors, with various other signal transduction pathways (Suresh, 2002).
Two forms of ER exist: ERα and ERβ. The former is a well-characterized mediator of estrogen-induced transcription, but the function of the latter is unclear and has necessitated a complete re-evaluation of estrogen signalling pathways in target cells.
ERα is expressed in ovary, uterus, vagina, mammary gland, and in central nervous system (CNS), whereas ERβ is expressed in the ovary, in the endometrium and myometrium, and is highly expressed in the CNS (Kuiper, 1997; Shughrue, 1998).
The cyclic growth and differentiation of the uterine endometrium and other tissues of the reproductive tract are controlled in large part by estrogen.
While the predominant ER in the uterus is ERα, numerous investigators have reported that ERβ is also expressed there, albeit at a markedly lower level (Kiper, 1997).
The differences in tissue and cell distribution of the two receptors are one of the factors underlying the tissue-specific response to estrogenic compounds.
There is relatively little known about the regulation of ERβ expression and role in target tissues. Most of the information regarding the role of these receptors stems from studies of transgenic mice in which either one or both receptor genes have been knocked out. Analysis of these animals led to the conclusions that ERβ is a modulator of ERα and has anti-uterotrophic effects, possibly by inhibiting the expression of IGF-I and down-regulation of progesteron receptor (PR) (Hall J.M, et al., 1999; Couse J.F et al., 2000). In the ovary, ERβ is down-regulated by gonadrotopin treatment (Byers et al., 1997), while estrogen has been reported to reduce ERβ expression in some areas of the brain and in the pituitary (Osterlend et al., 1998)
ERα and ERβ exhibit distinct interactions with common or specific cofactors, show differential binding to a variety of ligands, and may act by way of different response

elements, exhibiting altered specificity and affinity (Muramatsu, et al., 2000; Hall et al., 1999).

ERα and ERβ can form homo- or heterodimers (ERα/ERβ), and the formation of heterodimers is probably the reason for the inhibitory action of ERβ on ERα-induced transcription.

PROGESTERONE RECEPTOR

Progesterone (P4) is a key coordinator of female mammals, it directly regulates many functions, such as reproductive behaviour, uterine growth, and implantation. Most actions of P4 are mediated by the progesterone receptor (PR).

PR is a member of the steroid-retinoid receptor superfamily of ligand-activated transcription factors, which mediate genomic effects of gonadal steroids. In the most species, PR is composed of two major ligand-binding forms (the long B form and a N-terminal truncated A form) encoded by one gene (Graham JD. Et al. 1997).

Progesterone receptor is one of the best studied estrogen-regulated genes and is widely recognized as a marker for estrogen action. Regulation of the PR gene by estrogen has been extensively studied. Within the PR gene promoter, clusters of estrogen response element (ERE) half-sites are present, which are essential for transactivation of the PR gene by liganded ER (Kastner P. et al., 1990).

Since the PR protein level is thought to be a critical determinant of sensitivity to P4, almost all hormone treatment protocols designed to elicit effects of P4 usually involve previous estrogen priming to induce PR (Kurita et al., 2001).

INSULIN-LIKE GROWTH FACTOR-I

Insulin-like growth factor-I (IGF-I) is a single chain basic polypeptide of 70 amino acids with molecular mass of 7649 Da. (Rinderknecht et al., 1978). It has high structural similarity with pro-insulin, as its name indicates. Using molecular biology techniques it has been proven that IGFs are highly conserved proteins found in an array of vertebrates species.

IGF-I is an important growth factor that depends very much on the tissue: in the brain it has also differentiating effects.

IGF-I is produced in liver, bone cells and other tissues, stimulates somatic growth and is essential in cell growth and metabolism. Its serum levels are involved in pathogenesis of disease, such as diabetes, cardiovascular diseases, cancer and osteoporosis (Kajantie et al., 2003; Brixten et al., 1986). It is also known to have a broad range of effects including promotion of cell survival, stimulation of metabolism, proliferation and differentiation of cells. Levels are influenced by growth hormone (GH), insulin and nutrition, but these factors only partly explain the wide variation in the levels. Studies on the role of IGF-I have been hampered by the fact that circulating IGF-I levels do not necessarily reflect the local production of IGF-I in specific tissues, such as pancreatic beta cells, myocardium, bone cells or CNS. Over 90% of IGF-I is bound to specific IGF binding proteins, of which IGF-BP3 appears to be most frequent. A genetic polymorphism in the promoter region of IGF-I production (Rietveld et al., 2003). The receptor to this hormone is a tyrosine kinase.
Many compounds, including dyes, fluorides, etc. have been registered in patents for their capability to mimic the physiological activity of insulin or insulin-like growth factor (Amman et al., 1998). There is some evidence from experimental studies that IGF-I could be a mediator of the actions of E2 in the uterus (Richards et al., 1996).

1.8 Rat reproductive organs

The Prostate

The principal function of the prostate is to provide the proteins and ions that form the bulk of the seminal fluid.
The rodent prostate is composed of well defined lobes (dorsal, lateral and ventral), each with unique patterns of ductal branching and lobe-specific secretory proteins. These lobe-specific morphologic and functional differences appear to be induced by distinct subpopulations of mesenchyme within the urogenital sinuses. Dorsal and ventral portions have different inductive properties (Hayward et al., 2000).
Prostatic budding is initiated by prenatal androgenic action. Ductal branching morphogenesis, canalisation, and epithelial cytodifferentiation also require androgenic stimulation. In rodent models the majority of ductal branching morphogenesis occurs between birth and puberty when circulating androgen levels are low. At puberty there is a growth spurt characterized by an increase in prostatic wet weight but only a small increase

in the number of ductal tips (Sugymura et al., 1986). Data from Sugymura et al., indicate that the developing prostate is sensitive to low androgen titers for ductal branching morphogenesis and that its response to increased androgen levels at puberty (an increase in wet weight) is separate from the early ductal branching.

The physiological role of estrogens in the prostate is unclear; however, estrogens can give rise to squamous metaplasia (Walsh et al., 1997). Presumably, the estrogens influence prostate development and differentiation through ERs. In the rat, ERα has been localized by immunohistochemistry and by in situ hybridisation to periductal stromal cells and not to epithelial cells (Prins et al., 1997). ERβ is known to be expressed at high levels in epithelial cells of the rat prostate gland, but its regulation is not well known (Prins et al.,1998, Asano et al., 2003).

The Uterus

The uterus of the rat is classified as uterus duplex. The lumina of the uterine horns are completely separate and open as a paired external orifice. A partial fusion of the two horns occurs caudally in that they share a common outer longitudinal layer of myometrium (Anatomy of the laboratory rat).

Uterus is a major target tissue for ovarian hormones, it is composed of heterogeneous cell types (stromal, luminal, epithelial, glandular, epithelial, and smooth muscle) that undergo continuous syncronized changes of proliferation and differentiation in response to changes in levels of circulating estrogen and progesterone (Li S., 1994; Martin et al., 1997). All of the gonadal steroid hormone receptors, ERα, ERβ, PR and AR are expressed in this organ. Estrogen, by regulating estrogen target genes in a cell-specific manner, has different effects on different types of cell in the uterus. Proliferation is initiated at puberty in response to cycling estrogen, although the immature uterus is fully capable of responding to estradiol (E2), and estrogen induces both epithelial and stromal cell proliferation (Martin et al., 1973). Although ERα is highly expressed in the epithelium, proliferation of epithelial cells in response to E2 is thought to be indirect, i.e., growth factors secreted by stroma in response to estrogen are the mitogens in epithelium (Cooke P.S., et al.,1997) ERα plays an important role in differentiation by regulating target genes such as that for the progesterone receptor (PR) Both the stroma and epithelium express PR, and estrogen induces PR in the stromal and glandular epithelial cells, whereas it reduces it in the luminal epithelial cells (Tibbetts T. A. et al., 1998). The mechanism behind the cell specific

regulation of PR by estrogen remains unclear and could not be fully explained if all of the actions of estrogen in the uterus were mediated by ERα. The role of ERβ in uterus is not fully understood. ERβ can act as a transdominant repressor on ERα transcriptional activity at subsaturating concentration of E2 (Hall, J.M et al., 1999). One explanation for the inhibitory effects of ERβ on ERα function is that ERβ can form heterodimers with ERα which in turn regulate ER functions (Petersson, K., et al 1997; Cwley S.M., et al., 1997).

2. Aim of the Study

Polybrominated diphenyl ethers (PBDEs) are a novel class of environmental contaminants. They are bioaccumulated and biomagnified in the environment, and comparatively high levels are often found in aquatic biotopes from different parts of the world. An exponential increase of PBDE levels has been observed in Swedish mother's milk during a period from 1972 to 1997. Based on levels in food from 1999, the dietary intake of PBDE in Sweden has been estimated to be 0.05 µg per day. Since human milk is an indicator of the level of exposure of the maternal organism and hence of possible exposure of the fetus, and on the other hand, of the level of exposure of nursing infant, investigations of the developmental toxicity of PBDEs were considered to be necessary.

Up to now, knowledge on PBDE's effect has been quite limited. The main data gaps concern reproduction, developmental toxicity and carcinogenicity. Many international and national authorities realised the need for further investigations supporting several project s to identify more clearly the actions of PBDEs in mammals. The aim of this EU-funded study was to provide informations on developmental toxicity of PBDE. One of major components of commercial PBDE products, PBDE 99 (2,2',4,4',5-Pentabromo-diphenylether) which also is one of the most abundant congeners in human milk, was chosen.

The doses of PBDE 99 selected were 1 mg/kg/day (the LOAEL) and an high dose of 10 mg/kg/day. The 1 mg/kg was taken from preliminary results indicating developmental (behavioural) toxicity of PBDE 99 in mice at this dose level (Eriksson et al., 2001; Viberg et al., 2003)

Since PCBs have a similar structure to PBDE, Aroclor 1254 was selected as positive control.

The aim of the experiments conducted in our laboratory was to clarify possible effects of PBDE 99 (in comparison with PCB) on the development of reproductive organs at the organ level and gene expression level. Data on actions of PBDE on endocrine mechanisms were not conclusive, but interactions seemed possible, also in view of the structural relationship to PCBs.

3. Material and Methods

3.1. Chemicals

The chemicals tested were: 2,2',4,4',5-Pentabromodiphenylether (PBDE99) and an commercial mixture of polychlorinated biphenyls (PCB) Aroclor 1254, both provided by Promochem GmbH, Wesel, Germany. PBDE 99, as powder with purity > 99% was stored in a glass tube at room temperature and protected as much as possible from light exposure. The PBDE 99 solutions were prepared by dissolving PBDE in a few drops of Toluene (Scharlau) and adding a volume of sterile olive oil which also served as negative control. The toluene was being evaporated by a magnetic stirrer. Two different concentrations were prepared: 10mg of PBDE99 per 1 ml of olive oil and 1mg PBDE99/ 1ml olive oil.
Aroclor 1254 solutions (10mg/ml and 30mg/ml of olive oil) were prepared by mixing it with a volume of olive oil. All solutions were stored at +4°C.

3.2. Animals

The study was conducted on offspring of time-pregnant Long-Evans rats bred in our laboratory.
They were originally derived from Mollegard Breeding & Research Centre Denmark. Groups of 3-4 animals of the same sex were kept in (19 x 38 x 60 cm) plexigas cages housed in rooms with regular light/dark cycle (light on 02.00-16.00 h), controlled temperature and humidity (22 °C ± 1°C; 50%). They were fed with standard diet (Provimi Kliba AG, Kaiseraugst, Switzerland) and water ad libitum.

3.3. STEADY STATE LEVELS ANALYSIS (BASELINE ANALYSIS)

3.3.1 Experimental animals and prenatal exposure to the chemicals

The age of animals used for time-pregnant mating was a maximum of 6 months old for females and 12 months for males.
One receptive female was mated overnight with one male. The stage of pregnancy was defined as GD 1= 24 h after onset of mating.
Females exhibiting positive vaginal smears after time-pregnant mating and a consistent gain of weight till gestational day 9 (GD9) were housed in separate groups of two animals. They were treated with one daily injection (s.c.) from GD 10 till GD18 (9 injections) with PBDE 99 (1 or 10 mg/kg/day), or Aroclor 1254 (10 or 30 mg/kg/day) or vehicle control (olive oil), 0.1ml/ 100 g. body weight injection volume. At GD22 the pregnant females were separated.

3.3.2 Postnatal investigations

Offspring were investigated after birth for several developmental endpoints such as weight, righting reflex, ano-genital distance, and eye opening at fixed age points following a reprotox plan protocol. Mother behaviour was also observed. The litter size was adjusted to 8 - 10 pups per each litter in order to have comparable conditions between the treatment groups.

Reprotoxplan:

-Postnatal day (PN) 2: body weight, ano-genital distance, righting reflex, milk consumption, maternal behaviour, equalization of offspring number.
-PN4: body weight, righting reflex, milk consumption and maternal behaviour
-PN6: body weight, ano-genital distance, righting reflex, milk consumption, maternal behaviour and equalization of offspring number.
-PN8: body weight and righting reflex
-PN9: body weight, ano-genital distance, righting reflex and eye opening.
-PN14: body weight, ano-genital distance, righting reflex, eye opening, maternal behaviour and equalization.
-PN28: weaning from the mother.

-from PN34: puberty onset in females (vagina opening).
-fromPN41: puberty onset in males (preputial separation).

3.3.3 TISSUE PREPARATION

Animals at around 120 days of age were sacrificed by decapitation, the females always in metestrus=diestrus 1. Uterus, ventral and dorsal prostate were removed, weighed (wet weight), frozen with metylbuthane (Fluka) and stored in liquid nitrogen. Ovaries, testis and epididymis were weighed and stored (ovaries in 70% formalin, testis in ethanol, and epididymis at –80°C). Liver, coagulating glands and seminal vescicles were only weighed. Adipose tissue and serum were collected and stored at –20°C.

3.3.4 mRNA Quantification

Homogenization

The tissues being analysed for mRNA (uterus, ventral and dorsal prostate) were disrupted in a solution of RLT-puffer (Qiagen no.79216) and β-mercaptoethanol (Fluka) and homogenized by rotor (Politron-aggregate, Kinematica, Luzern CH). Ratio between RLT-buffer and β-mercaptoethanol: 1ml/10µl. Ratio between tissue weight and buffer 20mg/350µl. The homogenate was then centrifuged for 10 min at 3000 x g at 20 °C. After centrifugation, the supernatant was collected and stored at –80°C.

RNA extraction

Total tissue RNA was extracted following the handbook and using reagents from RNeasy Mini Kit (Qiagen cat. no. 74106) according to instructions by the company. Genomic DNA was thoroughly digested by DNAse-I (Qiagen).The concentration of RNA was determined by measuring the absorbance at 260 nm wave-length at several RNA dilutions. Pure RNA was stored in aqueous solution at –80°C till use.
The procedure steps were the following:

1. 350µl volume of clear supernatant was transferred into a clean tube and mixed with the same amount of Ethanol 70%.

2. The total mixture was applied to a Rneasy column, centrifuged for 15 sec at 11,000 g and the flow-through discarded.
3. 350µl RW 1 buffer was added to the column, centrifuged and flow-through discarded.
4. 80µl DNase I was added and incubated for 15 min at room temperature.
5. 350µl RW 1 buffer was added to the column, centrifuged, flow-through discarded and the column was transferred into a new clean tube.
6. The column was washed twice with RPE puffer with ethanol 70%.
7. At this point the column was eluted with 50µl of RNAse free water.

Reverse transcription

Reverse transcription of RNA to cDNA was performed by using TaqMan® Reverse Transcription Reagents kit according to manufacturer's instructions (Applied Biosystems N808-0234, Rotkreuz, Switzerland). The procedure followed is illustrated in the table below:

Table 3.1: Reverse Transcription reaction mix

RT component	1x100 µl	End concentration
H20	18.5	
10 x TaqMan RT buffer	10	1x
25 mM Magnesium Chloride	22	5.5 mM
DeoxyNTP's mixture	20	500 µM of each dNTP
50 µM RT primer*	5	2.5 µM
RNase Inhibitor	2	0.4 U/µl
MultiScribe TM Reverse Transcriptase	2.5	1.25 U/µl
RNA in H2O	20	10 ng/µl in total volume

*Random hexamers as primer was used.

The reverse transcription polymerise chain reaction was performed with the conditions shown in the table 3.2:

Table 3.2: RT cycle

Step	Incubation	Reverse Transcription	Reverse Transcription Inactivation	End
	Hold	Hold	Hold	Hold
Time (min)	10 min	30 min	5 min	
Temperature (°C)	25°C	48°C	95°C	4°C

At the end, the RT products (cDNA) were stored at –20°C.

Primer and Probe Sequence for real time PCR (5′⇒ 3′)

The gene sequence for primers and probes were designed by selecting an amplicon region from mRNA sequence (National Cancer for Biotecnology Information, gene bank) specific for each gene (AR, PR, IGF-1, ERα, ERβ and CycA) with PrimerExpress Software, Version 2.0. Each probe was labelled at 5′ end with FAM as reporter.

Androgen Receptor (AR) :

Forward Primer: 5'-CGGAAGGGAAACAGAAGTATCTATG-**3'**
TaqManProbe: 5'-CCAGCAGAAATGATTGCACCATTGATAAATTTC-**3'**
Reverse Primer: 5'-GGAGACGACACGATGGACAA-**3'**

Progesterone Receptor (PR):

Forward Primer: 5'-GCAATTGGCTTAAGACAGAAAGG-**3'**
TaqManProbe: 5'-CCCAGTTCACAACGCTTCTATCAACTTACAAAAC-**3'**
Reverse Primer: 5'-ACAAGATCATGCAAGCTGTCAAG-**3'**

Insulin Growth Factor-I (IGF-I):

Forward Primer: 5'-GGCCCAGCGCCACA-**3'**
TaqManProbe: 5'-TGACATGCCCAAGACTCAGAAGGAAGTACA-**3'**
Reverse Primer: 5'-TGTTTCCTGCACTTCCTCTACTTG-**3'**

Estrogen Receptor α (ERα):

Forward Primer: 5'-CCAAAGCCTCGGGAATGG-**3'**
TaqManProbe: 5'-TCGTTCCCTTGGATCTGGTGCAACAA-**3'**
Reverse Primer: 5'-AGCTGCGGGCGATTGAG-**3'**

Estrogen Receptor β (ERβ):

Forward Primer: 5'-TTGGTGTGAAGCAAGATCACTAGAG-**3'**
TaqManProbe: 5'-CCACTAAGCTTCCTCTTCAGTGTCTCTCTGTTTACA-**3'**
Reverse Primer: 5'-AACAGGGCTGGCACAACTG-**3'**

Cyclophillin A (Cyc):

Forward Primer: 5'-TGTGCCAGGGTGGTGACTT-**3'**
TaqManProbe: 5'-CCACCAGTGCCATTATGGCGTGT-**3'**
Reverse Primer: 5'-TCAAATTTCTCTCCGTAGATGGACTT-**3'**

They were ordered by Microsynth (Balgach, Switzerland) and diluted in H_2O, primers to 9000 nM and probes to 2250nM and stored at –20°C.

Real Time PCR reaction

For the mRNA quantification by Real Time-PCR, every reaction run was done on a 96 well plate with 25 µl of reaction-mix pro each well.
The reaction-mix components are listed in the scheme below:

Table 3.3: Real Time-PCR component mix.

Reagents	Volume/tube µl	End concentration
TaqMan Universal PCR Master Mix	12.5	1x
Forward primer	2.5	50-900nM
Reverse primer	2.5	50-900nM
TaqMan probe	2.5	25-225nM
DNA sample (cDNA from the investigated gene)	2.5	0.2-2ng/µl
Water	2.5	
Total volume	25	

TaqMan universal PCR Master Mix was purchased from Applied Biosystems as well as the plates. Probe, forward and reverse primers were ordered from Microsynth. Real Time-PCR was performed on the ABI PRISM 7700 Sequence Detector (Applied Biosystems).
The detecting run was composed of different step cycles which are described in the following scheme:

Table 3.4: detection run steps.

Thermal Cycler	Times and Temperatures			
	Initial Steps		Each of 50 Cycles	
			Melt	Anneal/Extend
ABI PRISM 7700 Sequence Detector	Hold	Hold	Cycle	
	2 min 50°C	10 min 95°C	15 sec 95°C	1 min 60°C

At the end of each run all the data were analysed using the Standard Curve Method.
A standard curve (cDNA diluted 1:2 four times) for the reference gene (cyc.) and for the gene of interest was included in each plate. mRNA concentration was calculated by plotting the Ct value to the respective standard curve.

3.4. ACUTE ESTROGEN CHALLENGE-STUDY

3.4.1 Experimental animals

Adult male and females Long Evans rats were obtained from Janvier (Le Genest St. Isle, France). Time-pregnant dams were treted with either 1mg/kg/ day or 10 mg/ kg/ day PBDE 99, 10 mg/ kg/ day Aroclor 1254, or vehicle from GD 10 until GD 18, and their offspring were raised until adulthood as described in the “Steady State” section.

3.4.2 Gonadectomy

At 10 weeks of age male and female F1 offspring were bilaterally gonadectomized under anesthesia to remove the primary source of sex hormones.

The following mixture was used for anesthesia:

Table 3.5: anesthesia components.

Components	Volume 10ml
Fentanyl citrate and fuanisone (Hypnorm Janssen)	1.25 ml
Medetomidine (Domitor Pfizer)	1.25 ml
Atropinsulfat 0.1% (Atropin Kantonsapotheke ZH)	0.25 ml
Tap Water	7.25 ml

Injection volume per each animal was: 150 µl/100g body weight.

3.4.3 Tissue preparation

Two weeks after gonadectomy the animals were given a single subcutaneous injection of 17-β Estradiol (E_2) (Calbiochem Lucerne, Switzerland). Vehicle controls were injected with 10µg/kg body weight dissolved in ethanol and then dimethylsulfoxide (DMSO, Fluka) or olive-oil.
Six hours after the injections, the animals were sacrificed by decapitation.
Uteri from females and ventral prostate from males were quickly removed, frozen with 2-methylbutane and stored in liquid nitrogen.

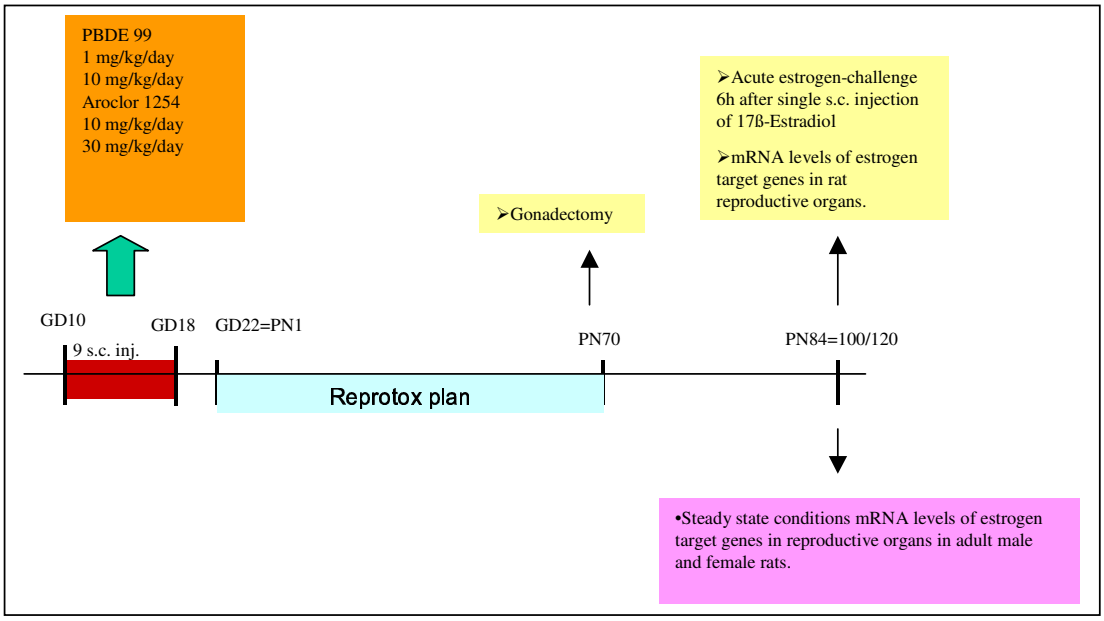
3.4.4 Determination of mRNA levels

The procedure followed was as used for the Steady State series. The only exception was the disruption of ventral prostate (vp) pieces, which was performed in a sonicator instead of homogenizer because of small size of the ventral prostate in gonadectomized animals.

3.5 STATISTICAL ANALYSES

All data were expressed as mean ± SD. The statistical analysis was done using SYSTAT Version 5.02 for Windows. All treatment groups were compared by one-way analysis of variance (ANOVA), followed by pair wise comparison by Bonferroni test.
Significant differences are indicated as following: $p < 0.05$ marked with: *, p range between 0.01 and 0. marked with **, $p < 0.001$ shown as ***.

3.6 Study design:



3.7 Synopsis of treatment groups:

Prenatal Treatment GD10→GD18	Sex	Adult Treatment
Steady State Study: • PBDE 1 mg/kg/day • PBDE 10 mg/kg/day • Aroclor 10 mg/kg/day • Aroclor 30 mg/kg/day • Vehicle	Male F1 Female F1	No adult treatment
Estrogen Challenge Study: • PBDE 1 mg/kg/day • PBDE 10 mg/kg/day • Aroclor 10 mg/kg/day • Vehicle	Male F1 Female F1	Gondectomy+ acute Vehicle Gonadectomy+acute10 µg/kg E2

4. Results

4.1 Samples and litter size

The total number of samples and litters per each treatment are listed in the table below, divided in males and females.

Male rat offspring:

Treatments	Number of animals	Number of litters
Control (C)	20	9
PBDE99 1mg/kg (P1)	14	7
PBDE99 10mg/kg (P10)	22	9
Aroclor 10mg/kg (Aro10)	20	5
Aroclor 30mg/kg (Aro30)	12	4

Female rat offspring:

Treatments	Number of animals	Number of litters
Control (C)	18	9
PBDE99 1mg/kg (P1)	12	7
PBDE99 10mg/kg (P10)	10	9
Aroclor 10mg/kg (Aro10)	13	5
Aroclor 30mg/kg (Aro30)	6	4

4.2 Surviving Rate

The surviving rate from post-natal day 2 (PN2) till PN 14 was not altered in treatment groups compared to control animals (fig. 4.1).

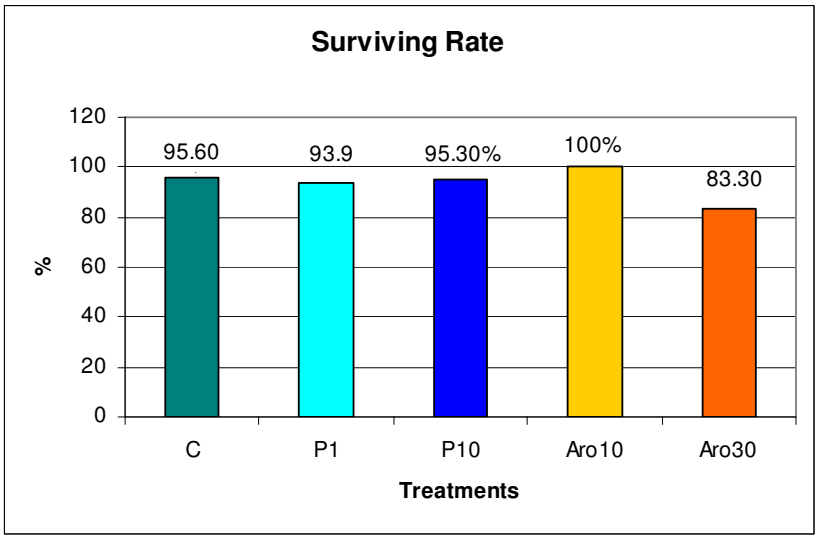


Figure 4.1: surviving rate from PN2 to PN14, in treatment groups: Control, PBDE99 1 mg/kg (P1), PBDE9910 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Data represent the % of all offspring per each treatment that reached PN14.

4.3 Onset of Puberty

The onset of puberty was checked by preputial separation in males and vagina opening in females. In males there was an early preputial separation in both PBDE 99 doses while in females there was a delay in PBDE 99 10 mg/kg dose group and in Aroclor 1254 30 mg/kg dose group (fig. 4.2).

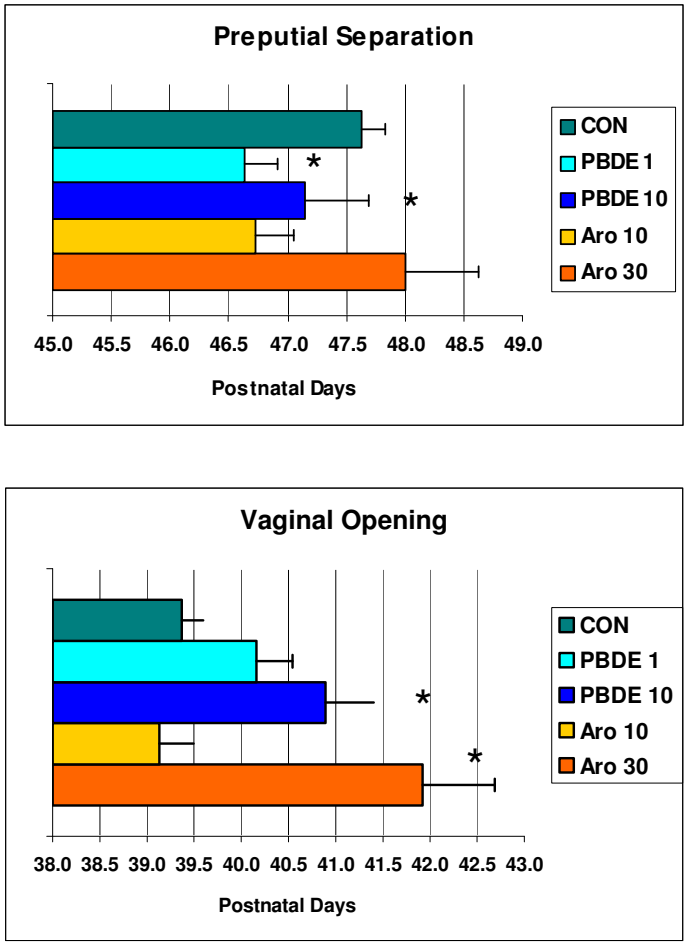


Figure 4.2: onset of puberty in male and female offspring in treatment groups: Control, PBDE99 1 mg/kg (P1), PBDE9910 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Data represent the post-natal days when preputial separation and vagina opening took place.

4.4 Body weight

The body weight of adult offspring studied at postnatal day (PN) 120 was not affected by prenatal treatment with PBDE99 (fig. 4.3). No significant change was observed. In females, there seemed to be a tendency toward a decrease in the low dose treatment group but the p value is > 0.05 (fig. 4.4).

Table 4.1: Body weight adult males.

Treatment	Mean (g)	S.D.	n
C	398.2	±29.3	20
P1	405.6	±25.3	14
P10	414.8	±28.4	22
Aro10	369.2	±31.9	20
Aro30	370.8	±61.5	12

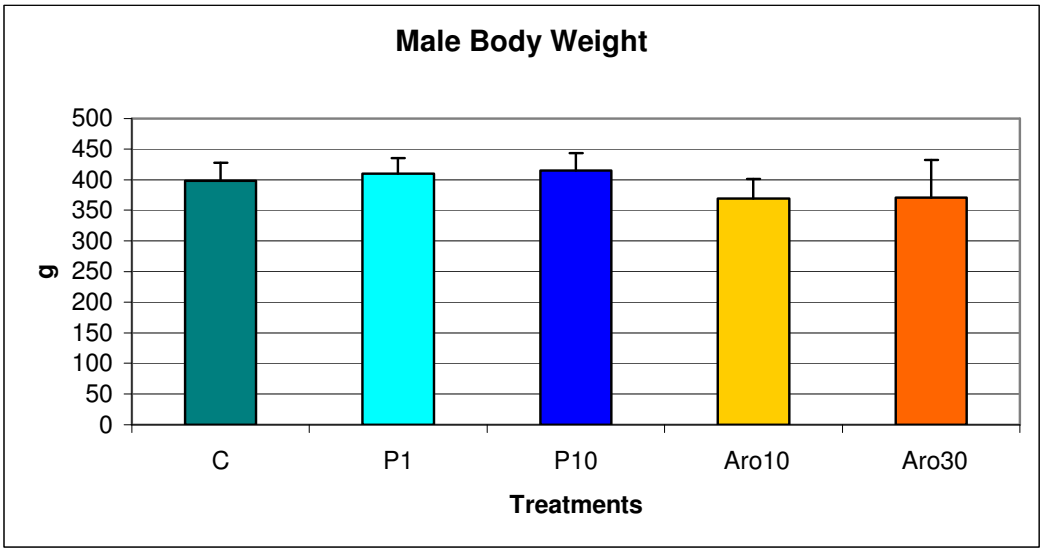


Figure 4.3: male adult body weight in different treatment groups: Control, PBDE99 1 mg/kg (P1), PBDE9910 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Data are represented as mean values ±S.D. and expressed in grams.

Table 4.2: Body weight adult females body weight.

Treatment	mean (g)	S.D.	n
C	244.2	±25.2	18
P1	229.2	±16.7	12
P10	240.8	±15.1	10
Aro10	226.9	±22.2	13
Aro30	178.4	±53.4	6

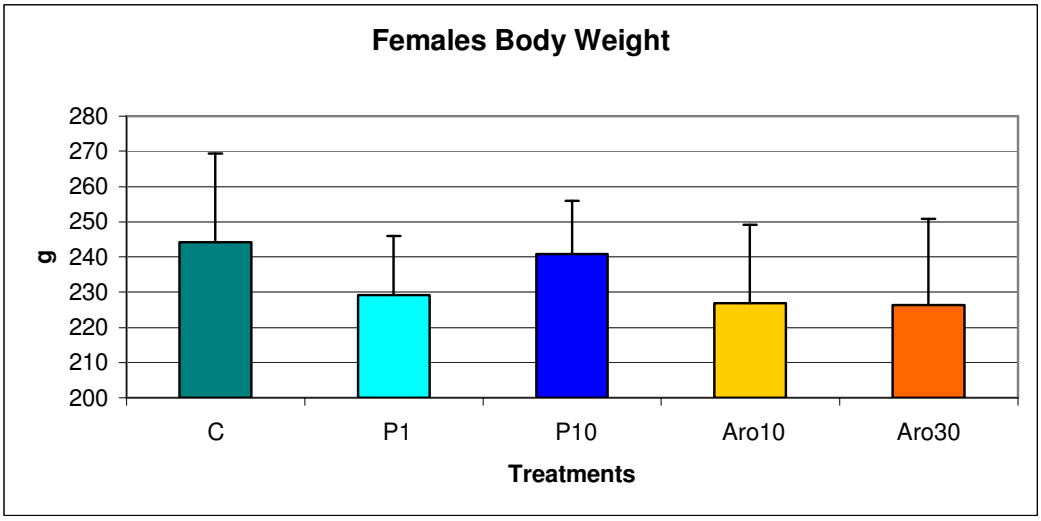


Figure 4.4: female adult body weight in different treatment groups: Control, PBDE99 1 mg/kg (P1), PBDE9910 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Data are represented as mean values ±S.D, and expressed in grams.

4.5 Organ weights

All organ wet weights were measured immediately after their removal. Absolute organ wet weight and organ wet weight normalized to body weight were considered. Asterisks indicate significant changes ($p<0.05$) compared to control group.

4.5.1 Males

Ventral Prostate

The absolute wet weight of ventral prostate did not show any relevant change. A significant increase was observed in relative weight (organ weight /body weight of the animal) in animals exposed to 1 mg/kg of PBDE 99 ($p = 0.011$) (fig. 4.6).

Table 4.3: ventral prostate weight.

	Absolute Weight		Relative Weight		
Treatment	**mean (g)**	**S.D.**	mean	**S.D.**	**n**
C	0.291	±0.082	0.000727	±0.000184	20
P1	0.368	±0.073	0.000916*	±0.00022	14
P10	0.310	±0.102	0.000773	±0.000213	22
Aro10	0.289	±0.067	0.000744	±0.000142	20
Aro30	0.138	±0.046	0.000475	±0.000362	11
* Different from Control p= 0.011					

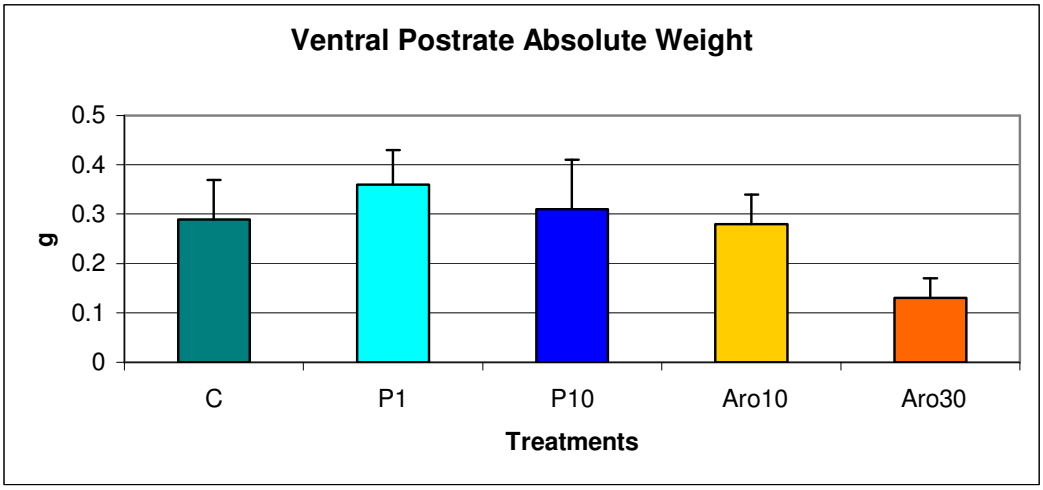


Figure 4.5: ventral prostate absolute wet weight mean in all treatment groups: Control, PBDE99 1 mg/kg (P1), PBDE9910 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Mean values ± S.D. are expressed in grams.

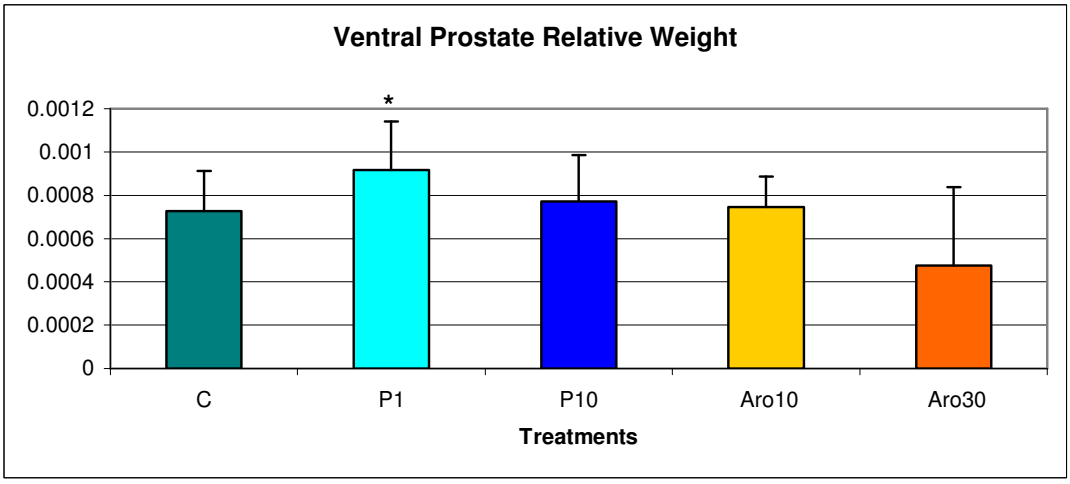


Figure 4.6: ventral prostate relative wet weight in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Ventral prostate wet weights (g) were normalized to animal body weight (g). Data represent mean ± S.D.

Dorsal Prostate

The absolute weight of dorsal prostate (fig.5) was found to be affected by exposure to the high PBDE 99 dose, with a significant increase (p=0.034). The same tendency was also observed in relative weight (fig. 4.8) with a p value on the borderline (p=0.05).

Table 4.4: dorsal prostate weight mean ±S.D.

	Absolute Weight		Relative Weight		
Treatment	**mean**	**S.D.**	mean	**S.D.**	**n**
C	0.259	±0.082	0.000651	±0.000208	20
P1	0.311	±0.069	0.000765	±0.000155	13
P10	0.318*	±0.089	0.000772*	±0.000213	22
Aro10	0.282	±0.061	0.000725	±0.000127	20
Aro30	0.197	±0.146	0.00053	±0.000402	12
* Different from Control with p< 0.05					

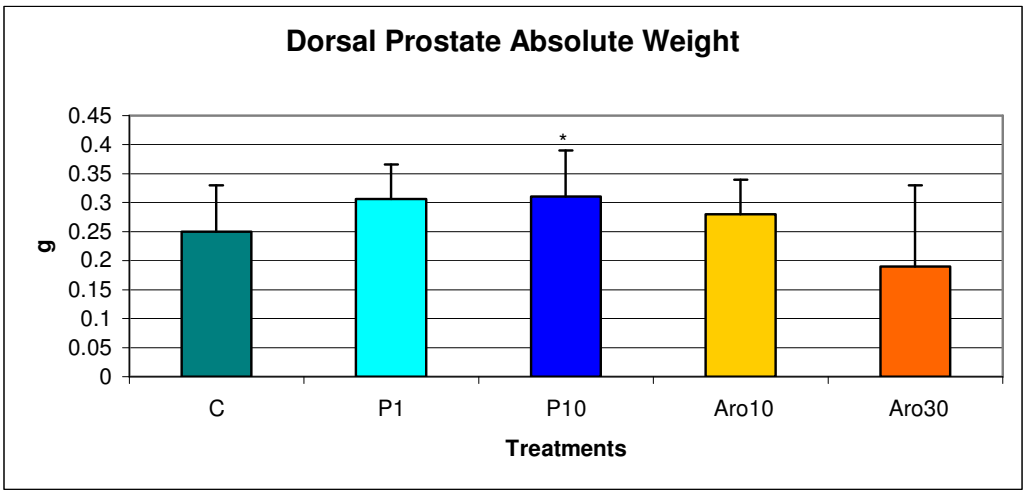


Figure 4.7: dorsal prostate absolute wet weights (g) in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Data are shown as mean ± S.D. Significant different from control * (p<0.05).

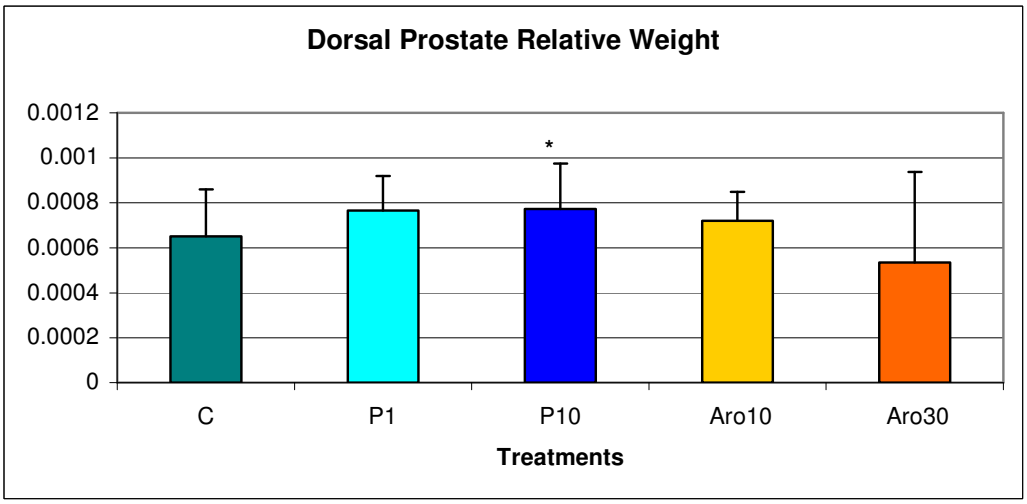


Figure 4.8: dorsal prostate relative wet weights in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Dorsal prostate wet weights (g.) were normalized to animal body weight (g). Data represent mean ± S.D. Significance against control is shown by * (p<0.05).

Testis

The combined weight of both testes did not show any changes absolute or relative weight of testis (fig. 4.9-4.10).

Table 4. 5: testis weight (g) (mean ±S.D).

	Absolute Weight		Relative Weight		
Treatment	**mean**	**S.D.**	mean	**S.D.**	**n**
C	3.11	±0.21	0.00783	±0.000588	20
P1	3.11	±0.18	0.00769	±0.000642	14
P10	3.14	±0.23	0.00768	±0.000933	21
Aro10	2.92	±0.51	0.00766	±0.0019	15
Aro30	3.06	±0.46	0.00827	±0.000933	12

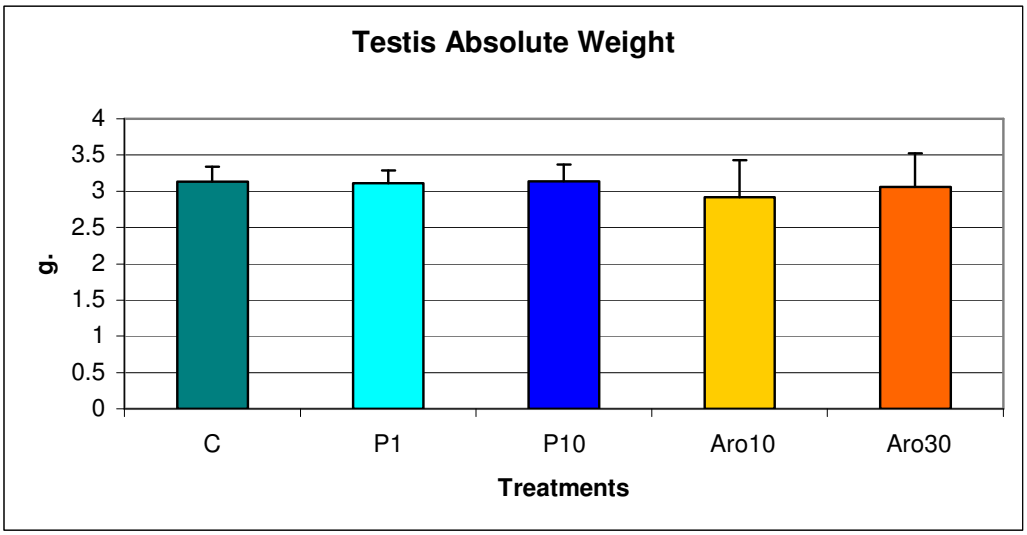


Figure 4.9: testis absolute combined weights (g) in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). The data represent the mean ± S.D. of all wet weight within every treatment group.

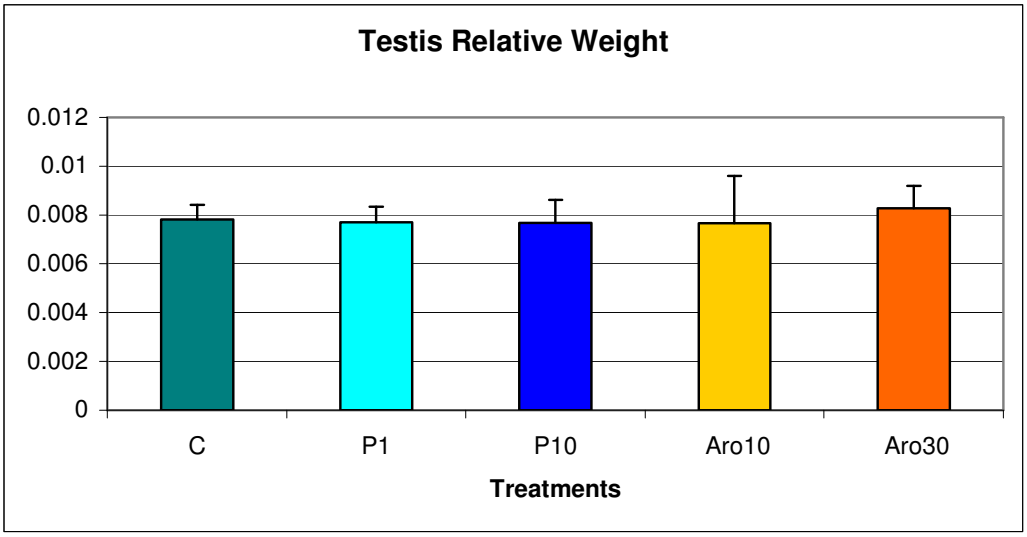


Figure 4.10: testis relative wet weight in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Testis wet weights (g) were normalized to animal body weight (g). Data represent means ± S.D.

Epididymis

The absolute combined weight of epididymis (fig. 4.11) was reduced in offspring of the low dose groups of PBDE99 (p=0.012) and Aroclor (p=0.016). The relative weight (fig. 4.12) showed a decrease with both PBDE 99 treatments with p values of 0.012 for P1 and 0.013 for P10.

Table 4.6: epidydimis combined weight (mean ± S.D).

	Absolute Weight		Relative Weight		
Treatment	**mean (g)**	**S.D.**	**mean**	**S.D.**	**n**
C	1.23	±0.22	0.0031	±0.00063	20
P1	1.08*	±0.08	0.0027*	±0.00022	14
P10	1.13	±0.14	0.0027*	±0.00037	22
Aro10	1.09*	±0.21	0.0028	±0.00066	20
Aro30	1.33	±0.22	0.0036	±0.00080	12
* Different from Control with p< 0.05					

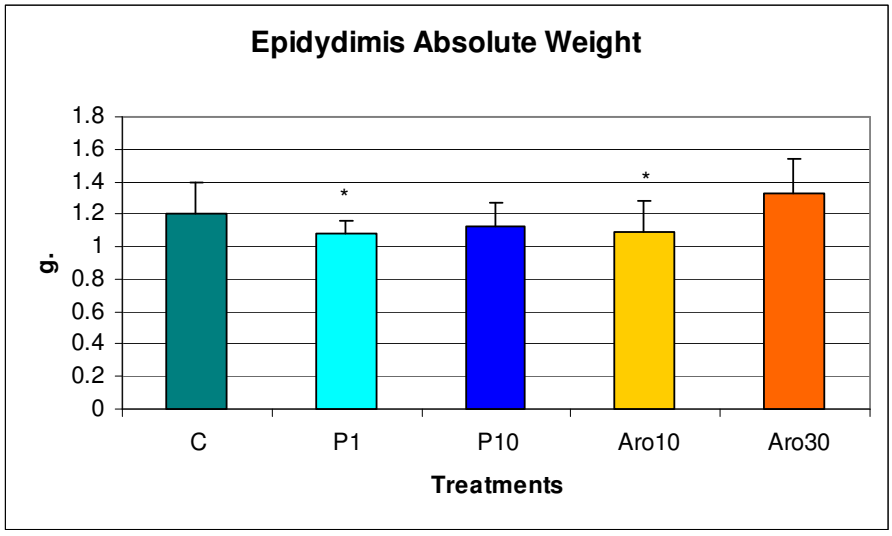


Figure 4.11: epididymis absolute wet weight (g) both epididymis combined in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30). Data represent mean ± S.D. of all wet weight within every treatment group. Significant difference from control shown by* ($p<0.05$).

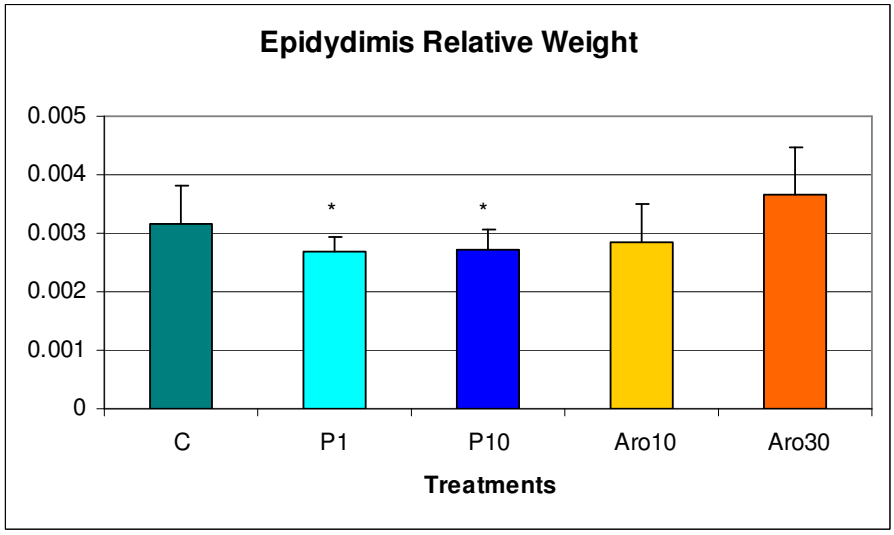


Figure 4.12: epididymis relative wet weight. The weights of both epididymis combined (g) in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro30. Epidymis wet weights (g.) were normalized to animal body weight (g). Data represent mean ± S.D. Significant differences from control are shown by * ($p<0.05$).

Coagulation Glands

The absolute combined coagulation glands weight (fig. 4.13) is affected with a decrease by Aro 10 exposure (p=0.000). The relative weight (fig. 4.14) did not show a significant increase.

Table 4.7: coagulation glands combined weights (mean ± S.D).

	Absolute Weight		Relative Weight		
Treatment	**mean (g)**	**S.D.**	**mean**	**S.D.**	**n**
C	0.1179	±0.06187	0.0002954	±0.0001554	20
P1	0.1221	±0.04600	0.0003012	±0.0001081	14
P10	0.122	±0.0204	0.0002968	±6.85E-05	18
Aro10	0.092***	±0.0407	0.0002131	±7.3326-05	17
Aro30	0.056	±0.02828	0.0001457	±6.654E-05	6
*** Extreme significant from Control (p<0.001)					

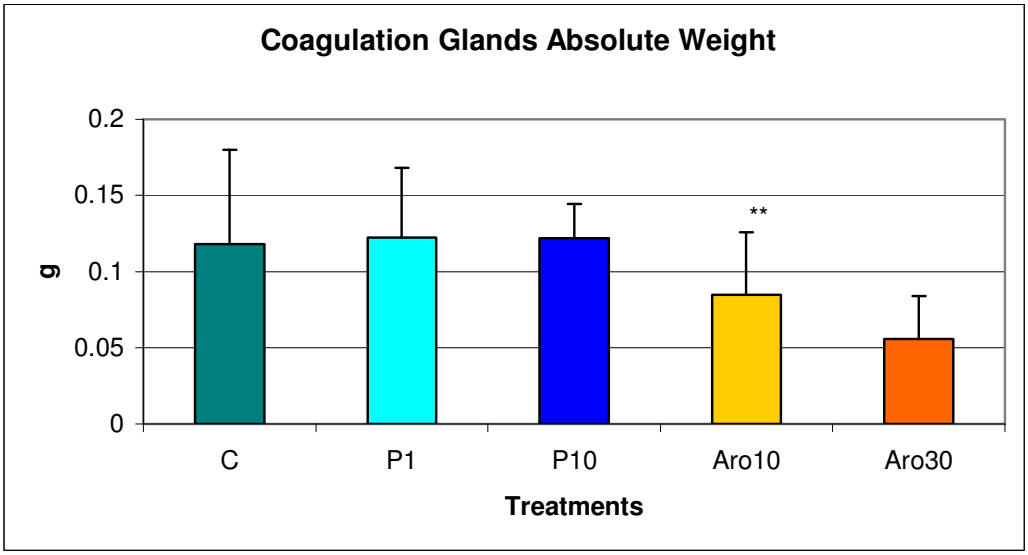


Figure 4.13: coagulation glands absolute wet weight (g). The weights of coagulation glands combined (g) in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). The data represent the mean ± S.D. of all wet weight within every treatment group. Extreme significances from control are shown by * (p<0.001).**

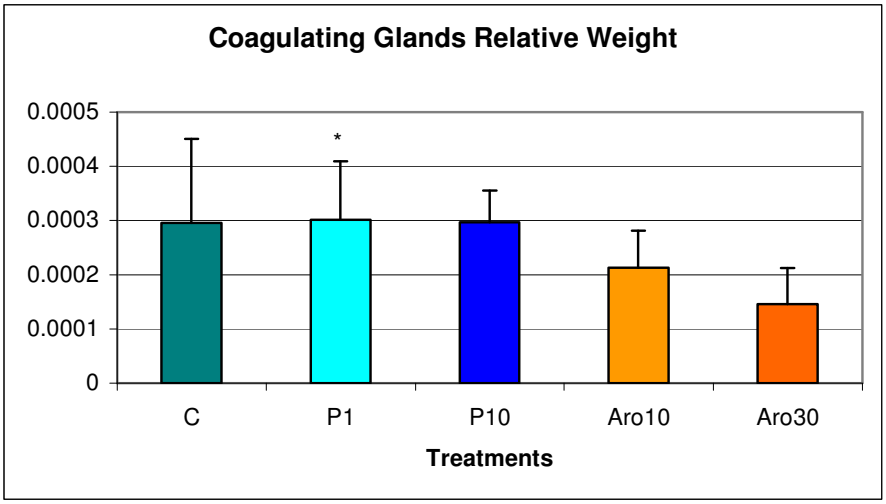


Figure 4.14: coagulation glands relative weight. The weights of coagulation glands combined (g) in all treatment groups: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Coagulation glands relative wet weights (g) were normalized to animal body weight (g). Data represent means ± S.D.

Seminal Vesicles

The absolute combined weight of seminal vesicles (fig.4.15) did not show any significant change after PBDE 99 exposure, while with Aro10 there was a highly significant increase in absolute as well as relative weight (p=0.000) (fig. 4.16).

Table 4.7: seminal vesicles combined weight (mean ± S.D).

	Absolute Weight		Relative Weight		
Treatment	**mean (g)**	**S.D.**	**mean (g)**	**S.D.**	**n**
C	0.948	±0.158	0.00238	±0.000367	20
P1	0.894	±0.252	0.00226	±0.000702	14
P10	0.706	±0.217	0.00190	±0.00054	21
Aro10	1.000***	±0.151	0.00248***	±0.000409	19
Aro30	0.977	±0.144	0.00234	±0.000317	11
*** Highly significant from control (p< 0.001)					

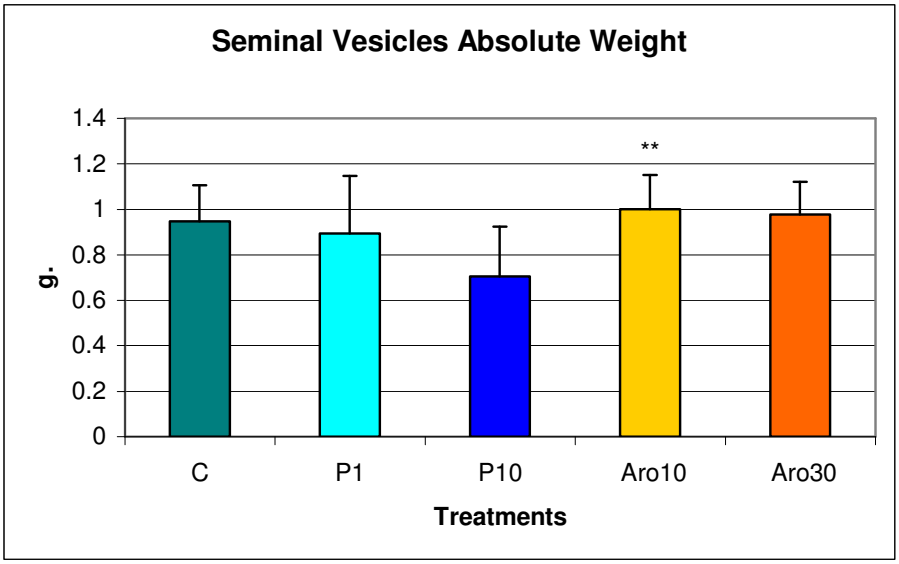


Figure 4.15: seminal vesicles absolute combined weight (g). The data represent the mean ± S.D. of all wet weight within every treatment group: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant differences from control are shown by* (p<0.001).**

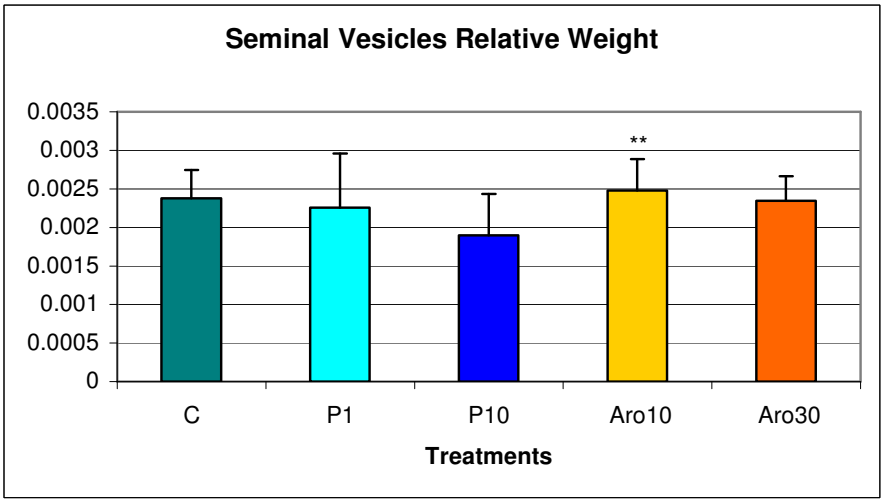


Figure 4.16: seminal vesicles combined relative wet weight (g). Seminal vesicles relative wet weights (g) were normalized to animal body weight (g). Data represent means ± S.D The data represent the mean ± S.D. of all wet weight within every treatment group: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Extremely significances are shown by * (p<0.001).**

Liver

Absolute and relative wet weight of liver did not show any relevant change (fig. 4.17-18).

Table 4.8: Liver wet weight (means ± S.D).

	Absolute Weight		Relative Weight		
Treatment	Mean (g)	**S.D.**	Mean	**S.D.**	**n**
C	13.3	±1.03	0.0337	±0.00267	20
P1	12.8	±1.23	0.0317	±0.000294	14
P10	12.9	±0.85	0.0311	±0.00246	22
Aro10	13.2	±2.21	0.0356	±0.00630	20
Aro30	13.2	±1.80	0.0356	±0.00273	12

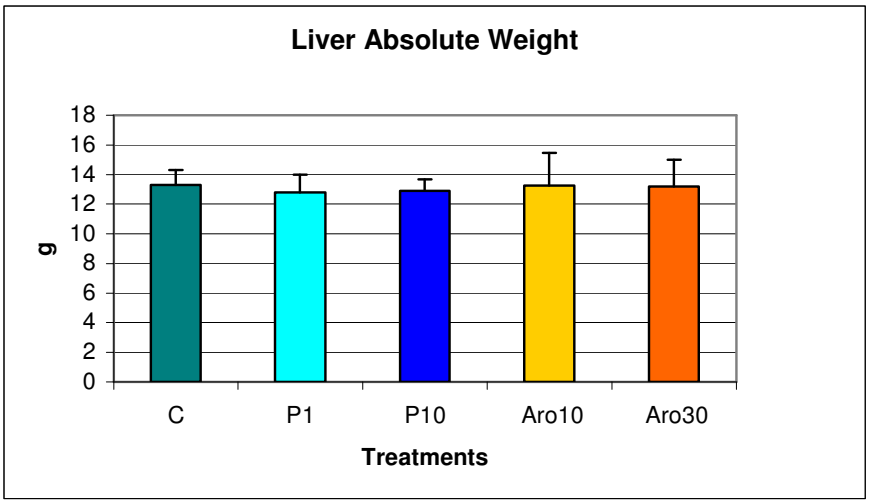


Figure 4.17: liver absolute wet weight (g). Data represent the mean ± S.D within every treatment group: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30).

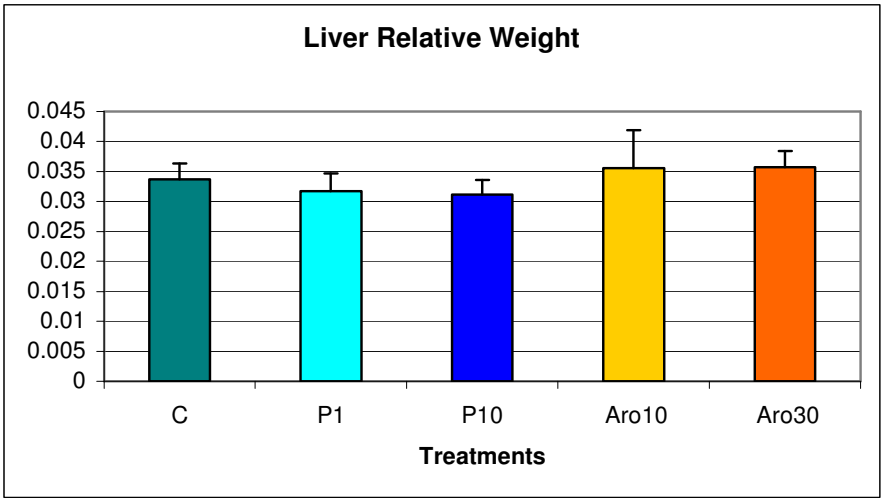


Figure 4.18: liver relative wet weight. Liver relative wet weights (g) were normalized to animal body weight (g) Data represent means ± S.D within every treatment group: Control, PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30).

Overview of organ weight in males

A general overview of the effects of PBDE99 and Aroclor 1254 on organ weights of adult offspring is given in the following schemes (scheme 4.1-2):

Absolute organ weight in male rat offspring:

	P1	P10	Aro10	Aro 30
Ventral Prostate				
Dorsal Prostate		↑		
Testis				
Epididymis	↓			
Coagulation glands			↓ ↓ ↓	
Seminal Vescicles			↑ ↑ ↑	
Liver				

Scheme 4.1: absolute wet weight (g) in males. Significant differences from control are indicated with arrows. The number of arrows indicates the significance level compared to the control group. Empty cells indicate no significant change.

Relative organ weight in male rat offspring:

	P1	P10	Aro10	Aro 30
Ventral Prostate	↑			
Dorsal Prostate		↑		
Testis				
Epididymis	↓	↓		
Coagulation glands	↑		↓ ↓ ↓	
Seminal Vescicles			↑ ↑ ↑	
Liver				

Scheme 4.2: relative organ weight in males. Wet weight was normalized to animal body weight. Significant differences from controls indicated by arrows. Their numbers indicate the significance levels compared to control treatment group. Empty cells indicate no significant changes.

4.5.2 Females

Uterus

The absolute weight of uterus at PN 120 did not show changes in PBDE 99 treatment groups, there was a significant decrease (p=0.007) in females exposed to Aroclor 10mg/kg (fig. 4.19).

The relative uterus weight (fig. 4.20) in PBDE 99 exposed groups also was not affected, while Aroclor 10mg/kg exhibited the same significant decrease (p=0.010).

Table 4.9: uterus weight (mean ± S.D).

	Absolute weight		Relative weight		
Treatment	**Mean (g)**	**S.D.**	**Mean**	**S.D.**	**n**
C	0.411	±0.112	0.00167	±0.000363	18
P1	0.429	±0.0202	0.00188	±0.0005	12
P10	0.424	±0.239	0.00173	±0.000558	10
Aro10	0.310*	±0.113	0.0013*	±0.00054	13
Aro30	0.247	±0.125	0.00133	±0.000466	6
* Different from control (p< 0.05)					

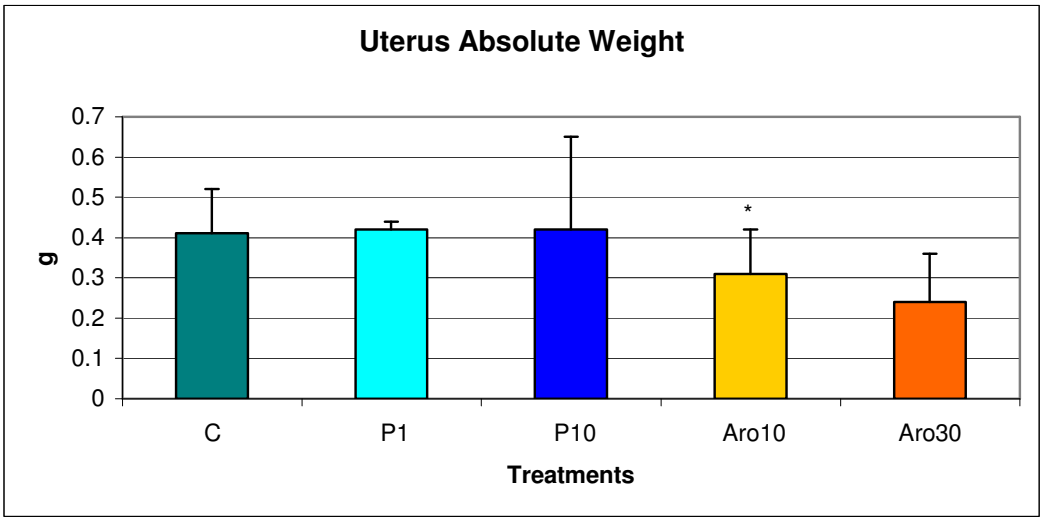


Figure 4.19: uterus absolute wet weight (g). Data represent mean ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant difference from control is shown by* (p<0.05).

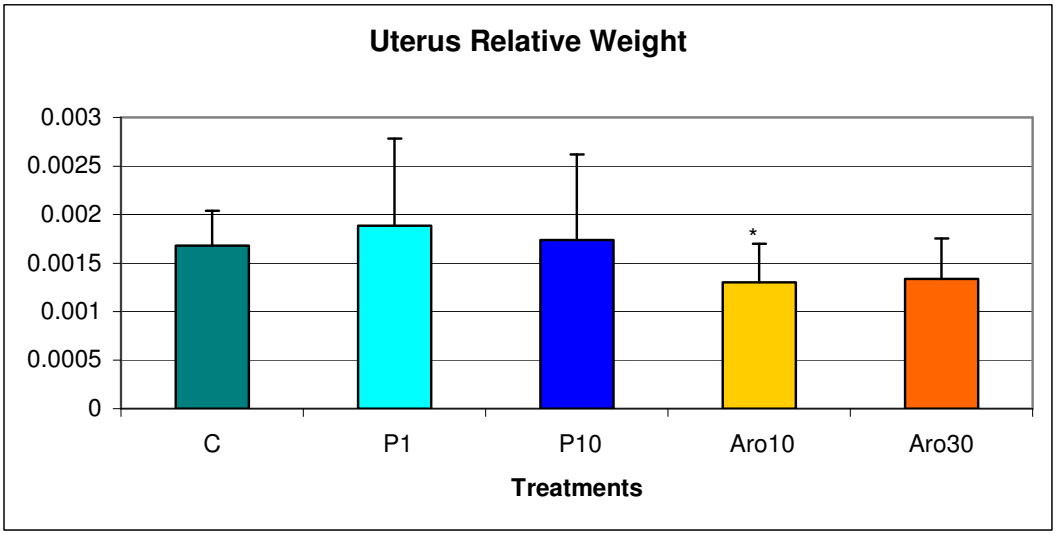


Figure 4.20: uterus relative wet weight (g). Uterus wet weights (g) was normalized to animal body weight (g). Data represent means ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant difference from control is shown by *(p<0.05).

Ovaries

The absolute weight of ovaries (fig. 4.21) did not show any significant change although the highest PBDE 99 concentration seemed to increase the weight with a p value on the borderline (p=0.053). Instead there were highly significant changes in relative weight (fig. 4.22). The P10 treatment group exhibited significant increase of relative weight (p=0.010), the data indicate a dose-dependent increase but the change in the P1 group was not significant. Aro 10 exposure also increased the relative weight (p=0.027).

Table 4.10: ovaries wet weight (mean ± S.D).

	Absolute weight		Relative weight		
Treatment	**Mean (g)**	**S.D.**	**Mean**	**S.D.**	**n**
C	0.113	±0.0226	0.000459	±0.000096	18
P1	0.113	±0.0208	0.000500	±0.000132	12
P10	0.135	±0.0265	0.000558*	±0.000086	10
Aro10	0.125	±0.0365	0.000544*	±0.00014	13
Aro30	0.089	±0.0174	0.000466	±0.000134	6
* Significant different from control (p<0.05)					

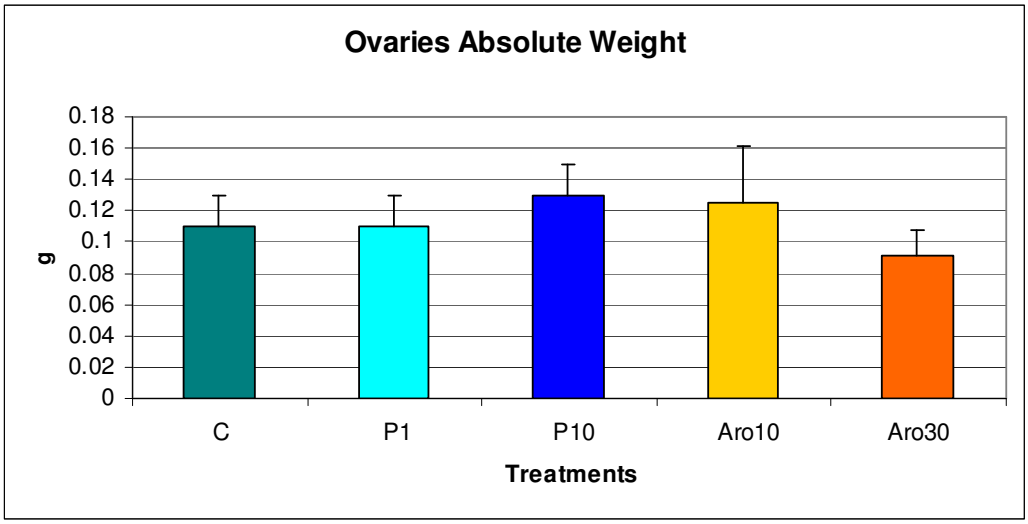


Figure 4.21: ovaries absolute wet weight (g). Data represent the mean ± S.D. of all wet weights within every treatment group: Control (C) PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant difference from control is shown by *(p<0.05).

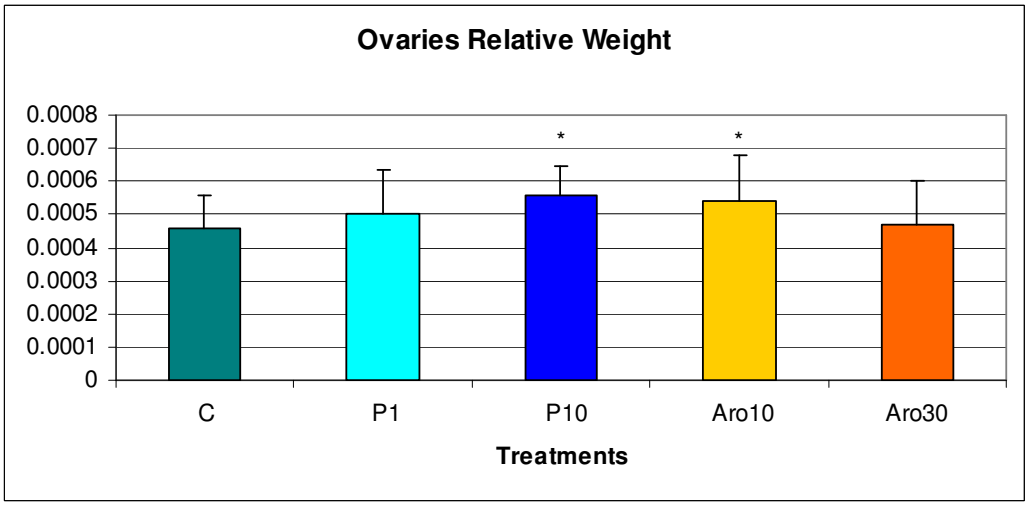


Figure 4.22: ovaries relative wet weight (g). Ovaries relative wet weights (g) were normalized to animal body weight (g). Data represent means ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant difference from control is shown by *(p<0.05).

Liver

No significant changes were observed in absolute liver weight (fig. 4.23). A significant increase of the relative weight was observed in females exposed to the higher dose of PBDE99 (p=0.026) (fig. 4.24).

Table 4.11: liver wet weight (mean ± S.D.)

	Absolute weight		Relative weight		
Treatment	**Mean (g)**	**S.D.**	**Mean**	**S.D.**	**n**
C	7.14	±0.540	0.02954	±0.00348	18
P1	6.91	±0.479	0.0303	±0.00335	12
P10	7.98	±1.355	0.0332*	±0.00551	10
Aro10	7.63	±1.888	0.0332	±0.00625	13
Aro30	6.41	±2.048	0.0359	±0.00817	6
* Significant different from control p<0.05					

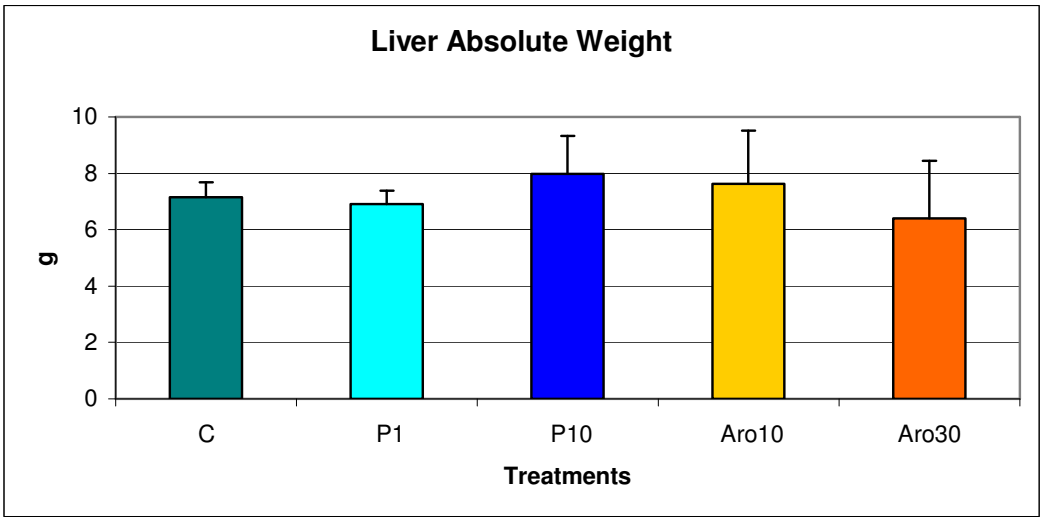


Figure 4.23: liver absolute wet weight (g). Data represent the mean ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30).

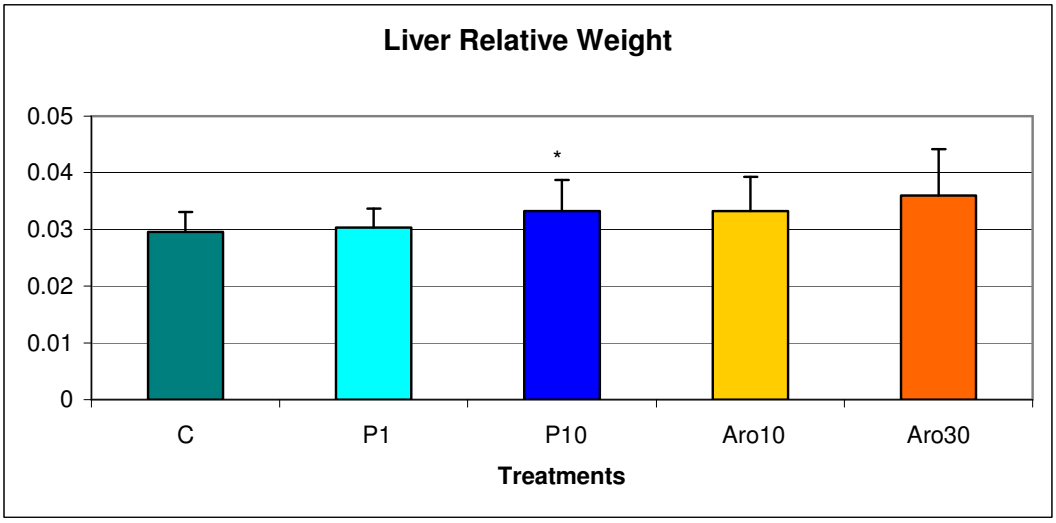


Figure 4.24: liver relative wet weight. Liver wet weight normalized with body weight. Data represent the mean ± S.D. within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant difference from control is indicated by a*(p<0.05).

Overview of organ weights in females:

A general overview of the effects of PBDE 99 and Aroclor 1254 on organ weight of adult females offspring is given in the following schemes (scheme 4.3-4):

Absolute organ weight in female rat offspring:

	P1	P10	Aro10	Aro30
Uterus			↓ ↓	
Ovaries				
Liver				

Scheme 4.3: absolute wet weight in females. Significant differences from control are shown by arrows. Their numbers indicate the significant level compared to control. Blank cells show no significant changes.

Relative organ weight organ weight in female rat offspring:

	P1	P10	Aro10	Aro30
Uterus			↓	
Ovaries		↑	↑	
Liver		↑		

Scheme 4.4: relative organ wet weight in females. The organ weight was normalized to animal body weight. Arrows show significant differences from control are. Their number indicates the significant levels compared to control. Empty cells indicate no significant changes.

4.6 mRNA Steady State Levels (Baseline)

The estrogen target gene mRNA levels were measured by real time-PCR in prostate, ventral and dorsal lobe, and in uterus of the same experimental groups where organ weights were determined (PN 120). In males, the genes studied were AR, ERα, ERβ and IGF-1. In females PR, ERα, ERβ and IGF-1 were investigated. The mRNA amount measured was normalized with Cyclophilin A (cyc) as reference gene. The number of samples was 9 (n=9) for each treatment from different litters (the same number used for organ weight determination). The Aroclor 30mg/kg samples were not analysed because of the reduced group size due to increased postnatal mortality. The animals exposed to this dose, in particular the females, hardly reached adulthood. Thus, changes in mRNA levels in the 30 mg/kg Aroclor group might have been due to non-specific toxic effects. Data represent the mean ± S.D.

4.6.1 Ventral Prostate

IGF-I

The Insulin-Like Growth Factor-I (IGF-I) mRNA level in ventral prostate was dose dependently reduced I after PBDE 99 exposure with significant difference from Control in both dose groups (p=0.01) in 1 mg/kg group, p=0.000 in 10 mg/kg (fig. 4.25). A significant reduction was also present after Aroclor treatment (p=0.006).

Table 4.12: IGF-I mRNA levels in ventral prostate normalized to cyclophillin (mean±S.D.)

Treatments	mean	S.D.	n
C	1.370	±0.380	9
P1	0.979*	±0.248	8
P10	0.806*	±0.255	9
Aro10	0.917*	±0.200	8
* Significant different from control p<0.05			

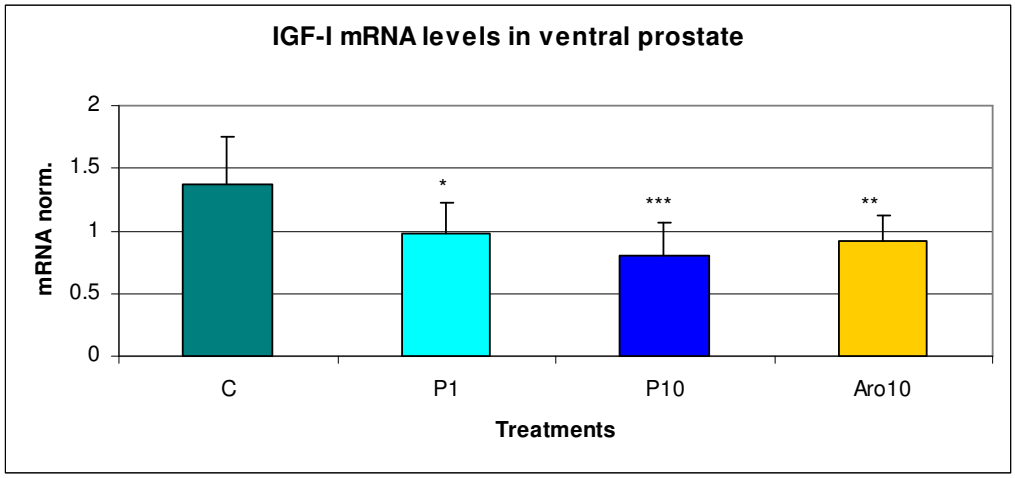


Figure 4.25: IGF-I mRNA levels in ventral prostate. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant differences from control are shown by asterisks (*p<0.05, **p<0.01 and ***p<0.001).

Androgen Receptor

The Androgen Receptor (AR) mRNA level in ventral prostate lobe shows a massive reduction in both PBDE 99 dosage groups (p=0.000) and was also decreased after Aroclor exposure (p=0.006) (fig. 4.26).

Table 4.13: androgen receptor mRNA levels in ventral prostate (mean ± S.D.).

Treatments	mean	S.D.	n
C	0.943	±0.116	7
P1	0.169***	±0.020	7
P10	0.189***	±0.044	7
Aro10	0.731**	±0.172	8
* Significant different from control p<0.05, **p<0.01 and ***p<0.001			

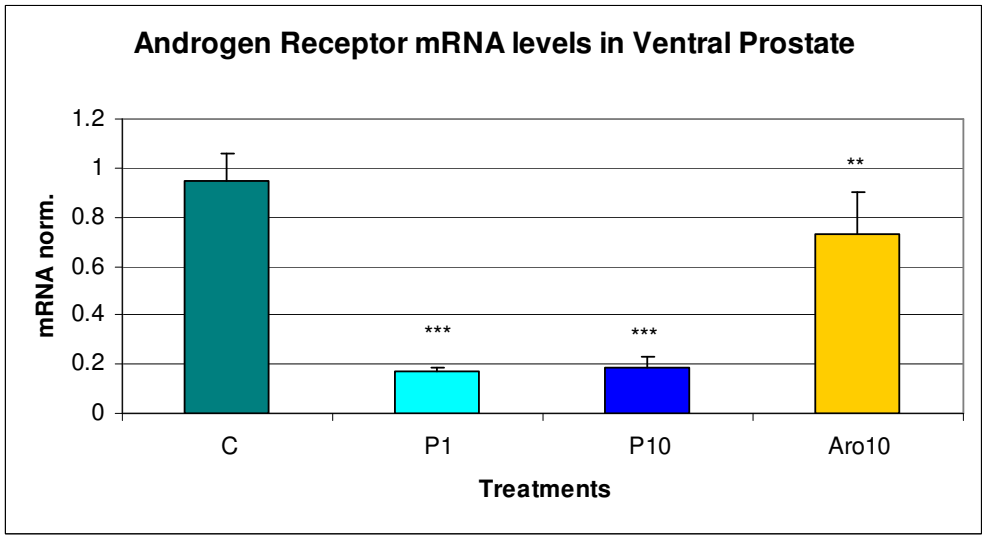


Figure 4.26: androgen receptor mRNA levels. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent the mean ± S.D. Data represent mean ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant differences from control are shown by asterisks (*p<0.05, **p<0.01 and ***p<0.001).

Estrogen Receptor α

Estrogen receptor α mRNA was difficult to quantify; in the P10 treatment group, the level of detection was 0.

Table 4.14: estrogen receptor α mRNA levels in ventral prostate (mean± S.D.).

Treatments	mean	S.D.	n
C	0.051	±0.019	7
P1	0.022	±0.01	7
P10	0.000***	±0.00	7
Aro10	0.029	±0.0066	6
*** Significant different from control p< 0.001			

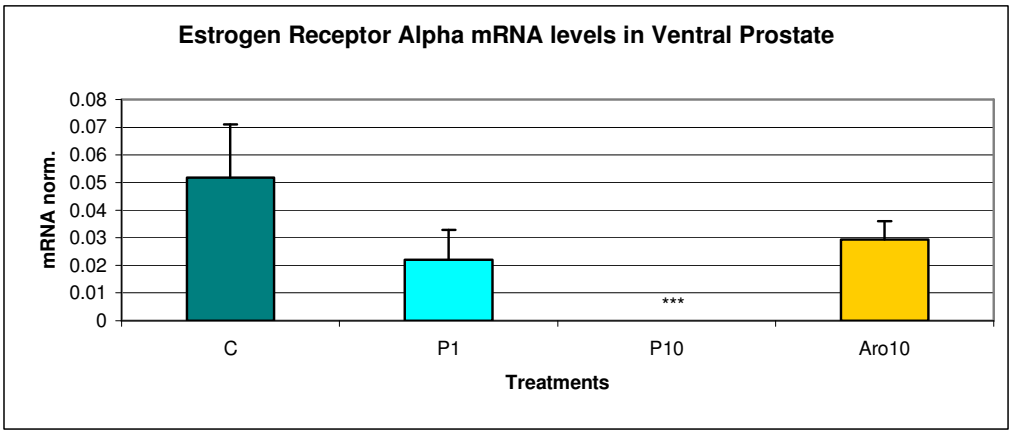


Figure 4.27: estrogen receptor α mRNA levels. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D. within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant differences from control are shown by asterisks (*p< 0.001).**

Estrogen Receptor β

The Estrogen Receptor β mRNA profile shows a significant decrease with a dose-dependent pattern in the two PBDE99 dose groups(low dose a p=0.015, high dose p=0.000). A significant decrease (p=0.021) was also found in the Aroclor group (fig. 4.28).

Table 4.15: estrogen receptor β mRNA levels in ventral prostate (mean± S.D.).

Treatments	mean	S.D.	n
C	1.481	±0.316	9

P1	0.978***	±0.348	8
P10	0.080***	±0.037	6
Aro10	1.011*	±0.366	9
* Significant different from control p<0.05, **p<0.01 and ***p<0.001			

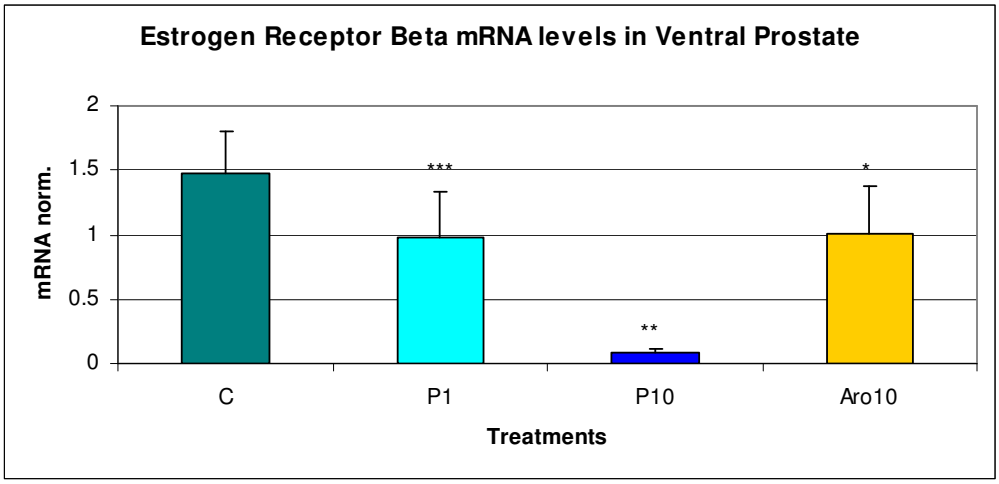


Figure 4.28: estrogen receptor β mRNA in ventral prostate. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D. within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), Aroclor 1254 10 mg/kg (Aro 10) and Aroclor 1254 30 mg/kg (Aro 30). Significant differences from control are shown by asterisks (*p<0.05, **p<0.01 and ***p<0.001).

4.6.2 Dorsal Prostate

IGF-I

IGF-I mRNA levels in dorsal prostate (fig. 4.29) were not affected by the exposure to PBDE 99, whereas Aroclor induced a significant increase (p=0.000).

Table 4.16: IGF-I mRNA levels in dorsal prostate normalized to cyclophilin (mean± S.D.).

Treatments	mean	S.D.	n
C	1.188	±0.287	9
P1	0.975	±0.185	9
P10	0.934	±0.092	9
Aro10	2.250***	±0.336	9
*** Significant difference from control p< 0.001			

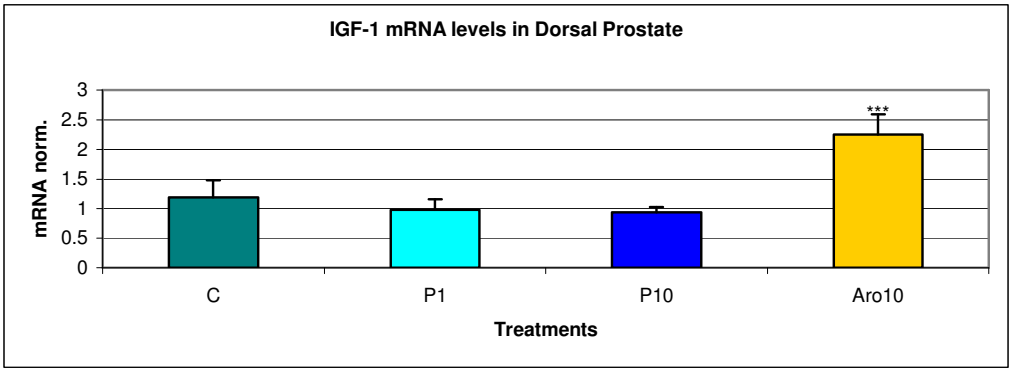


Figure 4.29: IGF-I mRNA levels in dorsal prostate. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D. within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), and Aroclor 1254 10 mg/kg (Aro 10). Significant differences from control are shown by asterisks (*p<0.001).**

Androgen Receptor

Androgen receptor mRNA expression in dorsal prostate (fig. 4.30) showed a dose-dependent increase in response to PBDE 99 exposure with p=0.000 in both dosage groups. Aroclor exposure did not affect AR mRNA levels.

Table 4.17: androgen receptor mRNA levels in dorsal prostate (mean± S.D.).

Treatments	mean	S.D.	n
C	0.6507	±0.103	9
P1	1.039***	±0.17	8
P10	2.1048***	±0.267	8
Aro10	0.481	±0.1097	9
*** Significant difference from control p< 0.001			

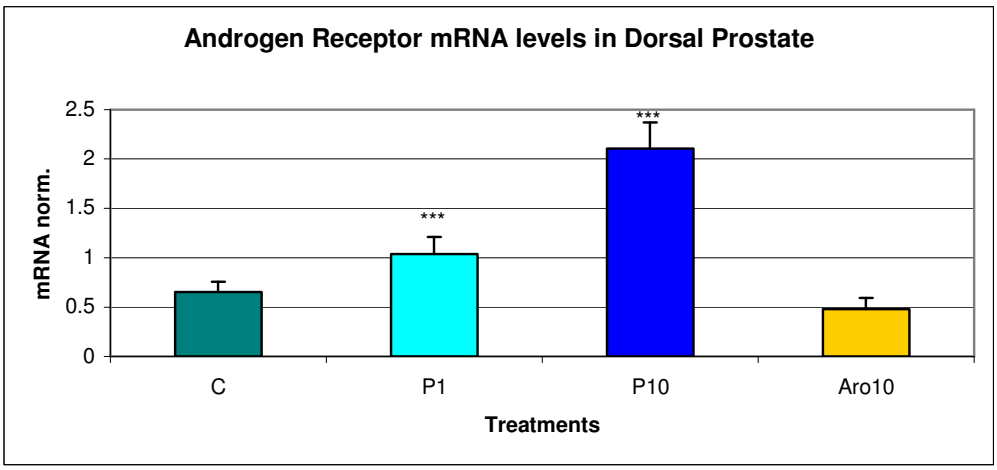


Figure 4.30: androgen receptor mRNA levels in dorsal prostate. mRNA was quantified by Real Time-PCR and normalized to cyclophilin . Data represent mean ± S.D. within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), and Aroclor 1254 10 mg/kg (Aro 10). Significant differences from control are shown by asterisks (*p<0.001).**

Estrogen Receptor α

Exposure to the high PBDE 99 dose (10 mg/kg) increased the level of ER α mRNA with p=0.000. 1 mg/kg PBDE 99 and Aroclor did not significantly affect the ER α mRNA level (fig. 4.31).

Table 4.18: estrogen receptor α mRNA levels in dorsal prostate (mean± S.D.).

Treatments	mean	S.D.	n
C	0.727	±0.183	7
P1	0.580	±0.151	9
P10	1.278***	±0.344	9
Aro10	0.610	±0.168	8
***Significant difference from control (p< 0.001)			

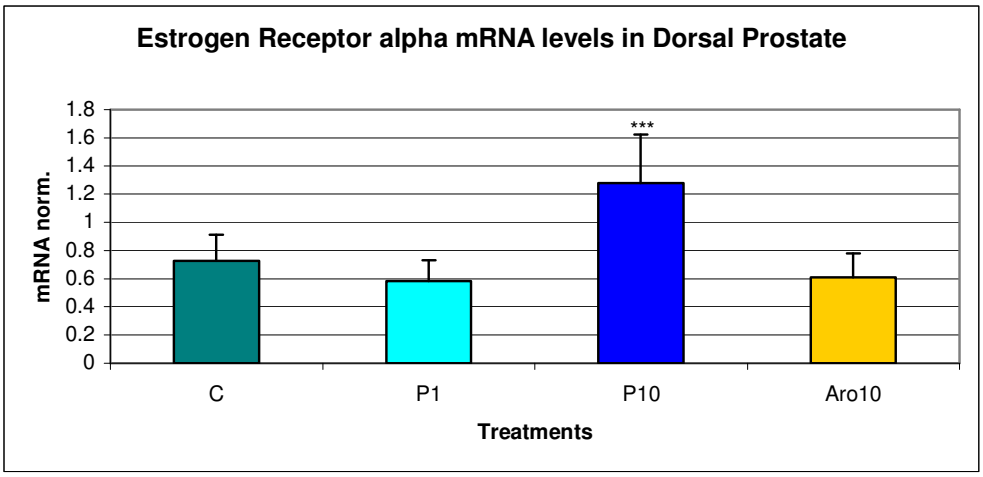


Figure 4.31: estrogen receptor α mRNA levels in dorsal prostate. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D. within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), and Aroclor 1254 10 mg/kg (Aro 10). Significant differences from control are shown by asterisks (*p<0.001).**

Estrogen Receptor β

Both PBDE 99 doses decreased ER β mRNA levels in dorsal prostate (fig. 4.32), with p value of 0.000. Aroclor was ineffective at the studied dose.

Table 4.19: estrogen receptor β mRNA normalized in dorsal prostate(mean± S.D.).

Treatments	mean	S.D.	n
C	0.829	±0.264	9
P1	0.430***	±0.112	9
P10	0.422***	±0.128	9
Aro10	1.057	±0.146	7
***Significant difference from control (p< 0.001)			

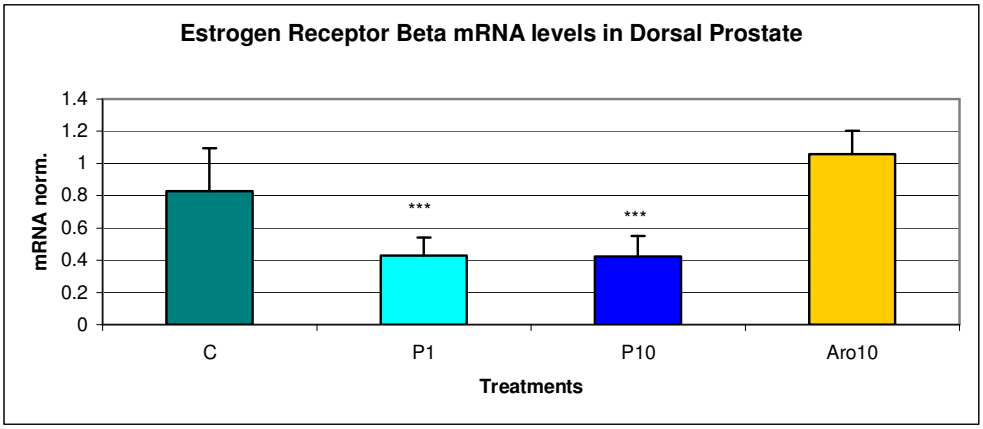


Figure 4.32: estrogen receptor β mRNA levels in dorsal prostate. mRNA amount was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D. within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), and Aroclor 1254 10 mg/kg (Aro 10). Significant differences from control are shown by asterisks (*p<0.001).**

4.6.3 Uterus

IGF-I

The IGF-I mRNA level in uterus (fig. 4.33) did not exhibit any significant change after PBDE 99 Aroclor 1254 exposure.

Table 4.21: IFG-I mRNA normalized in uterus (mean± S.D.).

Treatments	mean	S.D.	n
C	0.177	±0.065	11
P1	0.345	±0.051	6
P10	0.219	±0.093	6
Aro10	0.378	±0.231	7

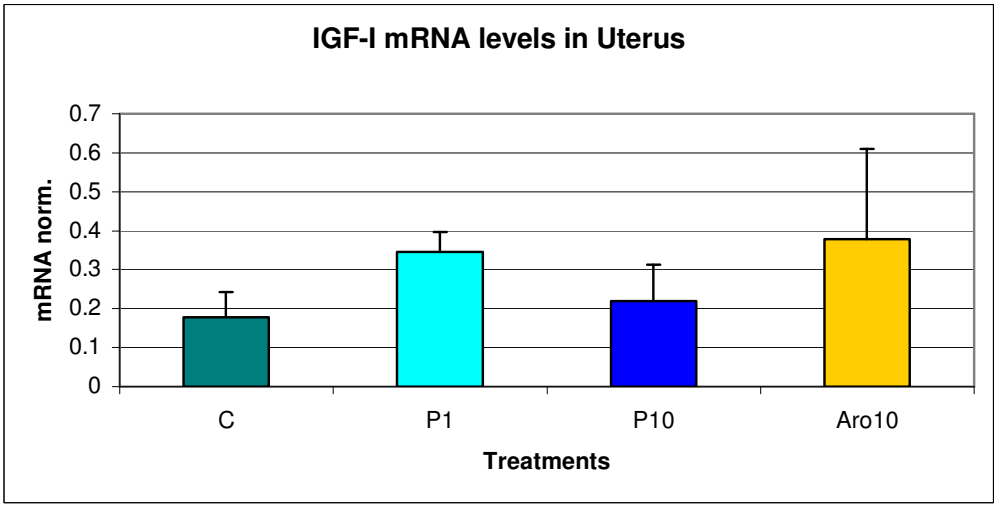


Figure 4.33: IGF-I mRNA levels in uterus. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D. within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), and Aroclor 1254 10 mg/kg (Aro 10).

Progesterone Receptor

PBDE 99 treatment affected the PR mRNA level in uterus (fig. 4.34) by a significant dose dependent decrease that reached statistical significance at both doses (low dose treatment (P1) p=0.004, high dose (P10) p=0.01).

Table 4.21: progesterone receptor mRNA levels in uterus (mean± S.D.).

Treatments	Average	S.D.	n
C	1.110	±0.238	10
P1	0.833	±0.400	8
P10	0.553	±0.181	8
Aro10	0.842	±0.439	9
*Significant difference from control (p<0.05), **(p<0.01)			

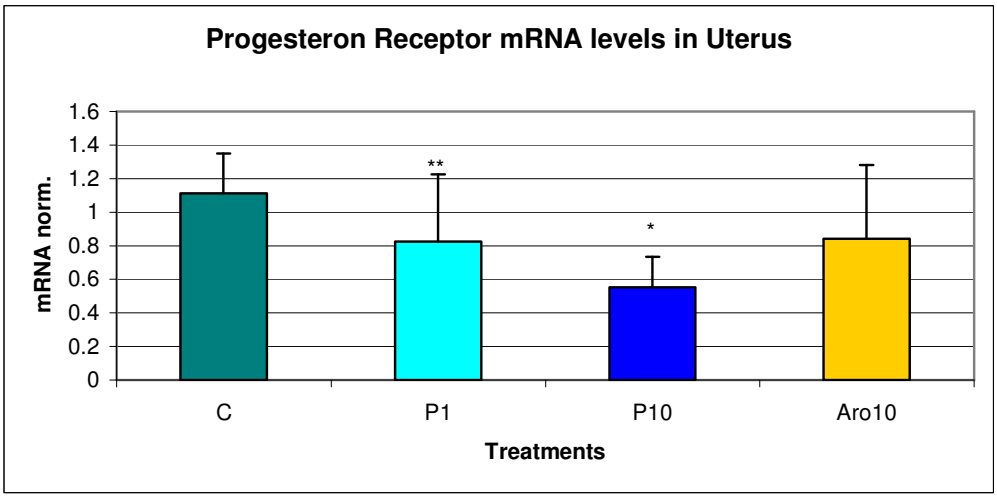


Figure 4. 34: progesterone receptor mRNA levels in uterus. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), and Aroclor 1254 10 mg/kg (Aro 10). Significant differences from control are shown by asterisks (*p<0.05, **<p<0.01).

Estrogen Receptor α

Estrogen receptor α m RNA levels were not significantly affected by exposure to PBDE99 or Aroclor 1254. The apparent increase in the low dose PBDE 99 dose group was not significant.

Table 4.22: estrogen receptor α mRNA levels in uterus (mean± S.D.).

Treatments	Average	S.D.	n
C	1.313	±0.375	8
P1	1.855	±0.566	9
P10	1.493	±0.671	6
Aro10	0.7503	±0.228	6

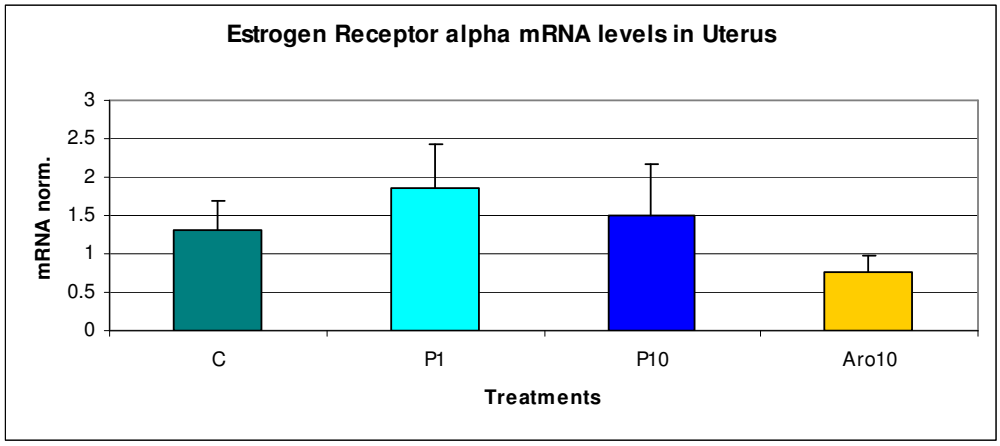


Figure 4. 35: estrogen receptor α mRNA levels in uterus. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), and Aroclor 1254 10 mg/kg (Aro 10).

Estrogen Receptor β

The effect of PBDE 99 on ER β mRNA levels was bell-shaped with an increase (p=0.000) after 1 mg/kg and a decrease (p=0.005) after 10 mg/kg. In the Aro 10 treatment group the level was 0.

Table 4.23: estrogen receptor β mRNA levels in uterus (mean± S.D.).

Treatments	Average	S.D.	N
C	0.00293	±0.00110	8
P1	0.00587***	±0.00148	7
P10	0.00122**	±0.00025	6
Aro10	0.00000	±0.00000	9
Significant difference from control(p<0.01), *(p<0.001)			

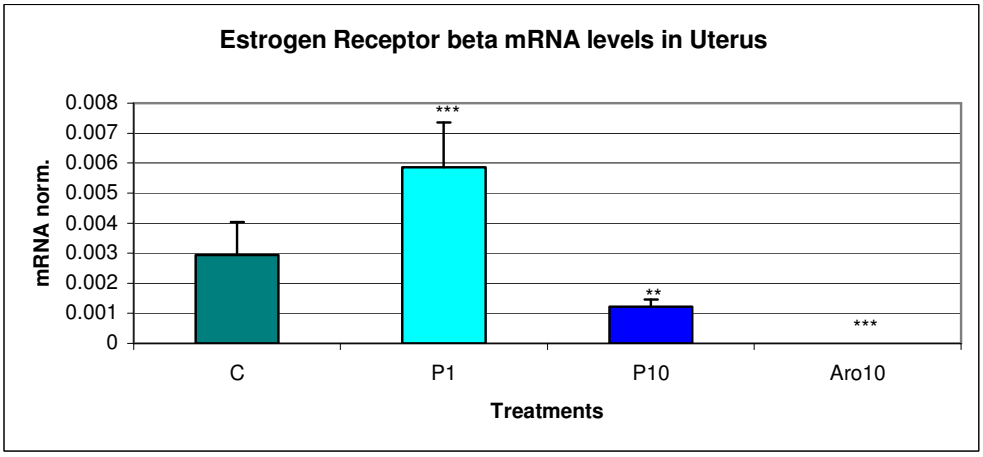


Figure 4.36: estrogen receptor β mRNA levels in uterus. mRNA was quantified by Real Time-PCR and normalized to cyclophilin. Data represent mean ± S.D within every treatment group: Control (C), PBDE 99 1 mg/kg (P1), PBDE 99 10 mg/kg (P10), and Aroclor 1254 10 mg/kg (Aro 10). Significant differences from control are shown by asterisks (p<0.01 and ***p<0.001). Cases of no detection are indicated by N.D.**

General overview on mRNA expression levels

The following schemes give a general overview on effects of PBDE 99 and Aroclor 1254 on gene expression in ventral prostate, dorsal prostate and uterus:

Ventral Prostate:

	P1	P10	Aro10
IGF-I	↓	↓↓↓	↓↓
AR	↓↓↓	↓↓↓	↓↓
ERα	↔	↓↓↓	↔
ERβ	↓↓↓	↓↓↓	↓

Scheme 4.5: Ventral prostate mRNA expression. The arrows indicate significant changes. Their number is proportional to the level of significance. Horizontal arrows indicate no significant change.

Dorsal Prostate:

	P1	P10	Aro10
IGF-1	↔	↔	↑↑↑
AR	↑↑↑	↑↑↑	↔
ERα	↔	↑↑↑	↔
ERβ	↓↓↓	↓↓↓	↔

Scheme 4.6: Dorsal prostate mRNA expression. The arrows indicate significant changes. Their number is proportional to the level of significance. Horizontal arrows indicate no significant change.

Uterus:

	P1	P10	Aro10
IGF-I	↔	↔	↔
PR	↓↓	↓	↔
ERα	↔	↔	↔
ERβ	↑↑↑	↓↓↓	↓↓↓

Scheme 4.7: Uterus mRNA expression. The arrows indicate significant changes. Their number is proportional to the level of significance. Horizontal arrows indicate no significant change.

4.7. mRNA Levels after Acute Estrogen Challenge

This study was conducted to examine the sensitivity of estrogen target genes to estrogen in adult developmentally chemical-exposed offspring. A single subcutaneous injection of Estradiol (E2) (10µg/kg of body weight) was given to adult gonadectomized male and female offspring. After 6 hours, tissues were analysed.

In two separate experimental series, two different vehicles were used: Dimethylsufoxide (DMSO) and olive oil in order to assess whether the acute effect of E2 depended on the vehicle.

mRNA levels quantified by Real Time PCR were: IGF-1, AR, PR, ERα and ERβ in ventral prostate and uterus. Animals from every treatment group (C, P1, P10 and Aro10) were gonadectomized at PN 70 and injected with E2 or with vehicle (DMSO or Olive-oil) on PN 84. The animal number per treatment group (vehicle or estradiol) was n=9-7 from 3 different litters per treatment in analyses with DMSO, in analyses with olive oil as vehicle the litter number was: 8 for Control, 4 for PBDE 99 1 mg/kg, 6 for PBDE 99 10 mg/kg, and 7 for Aroclor 1254 10 mg/kg.

4.7.1 Acute Challenge Study with DMSO as Vehicle

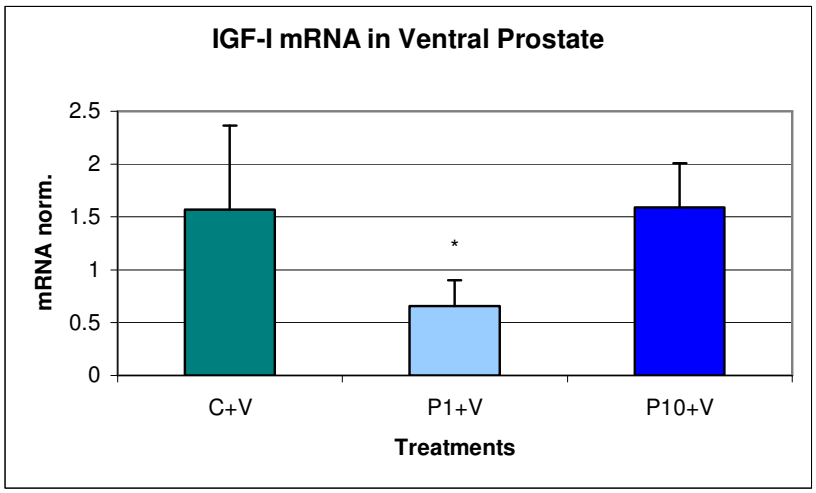
The analyses were done on animals prenatally exposed to PBDE99 1 mg/kg body weight and 10 mg/kg body weight and on controls, gonadectomized and injected with 10µg/kg E2 in adulthood.

I. mRNA levels in acute vehicle-injected gonadectomized rats of different treatment groups

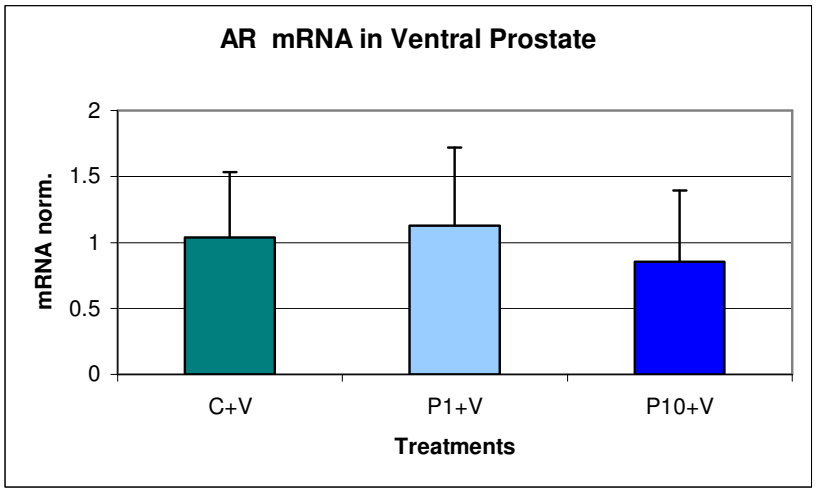
Ia Ventral Prostate

Ventral prostates of adult offspring gonadectomized at 10 weeks of age, sacrificed at week 12 and exposed to Vehicle (DMSO,) were analysed for their mRNA levels of IGF-I, AR, ERα and ERβ. Chemical-exposed, vehicle-injected groups were compared with untreated, vehicle-injected controls. As compared to controls, a significant decrease in IGF-I mRNA levels were observed in the PBDE 99 low dose group. The other genes were not significantly different from gonadectomized controls (fig. 4.37). In intact offspring studied under steady state conditions (fig. 4.25) the same tendency was observed in IGF-I and ER β although there were no significant changes. ERα mRNA levels were very low in both intact and gonadectomized rats.

IGF-I mRNA in Ventral Prostate

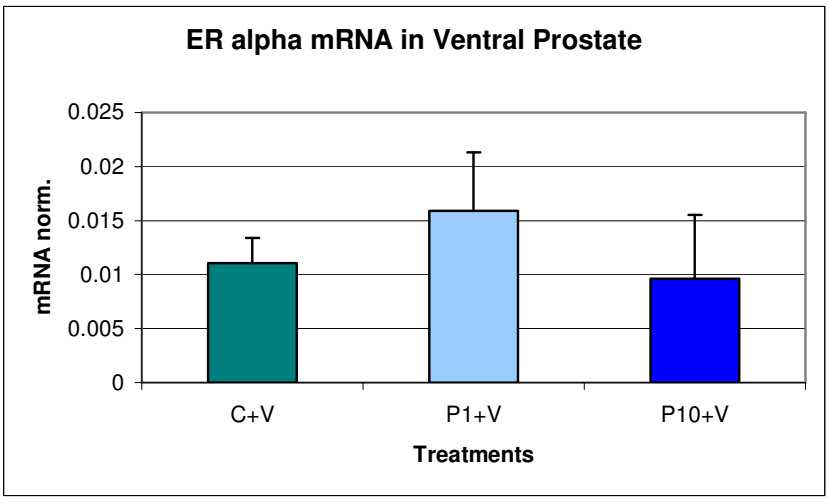


AR mRNA in Ventral Prostate



C. ER alpha in Ventral Prostate

ERα mRNA in Ventral Prostate



ERβ mRNA in Ventral Prostate

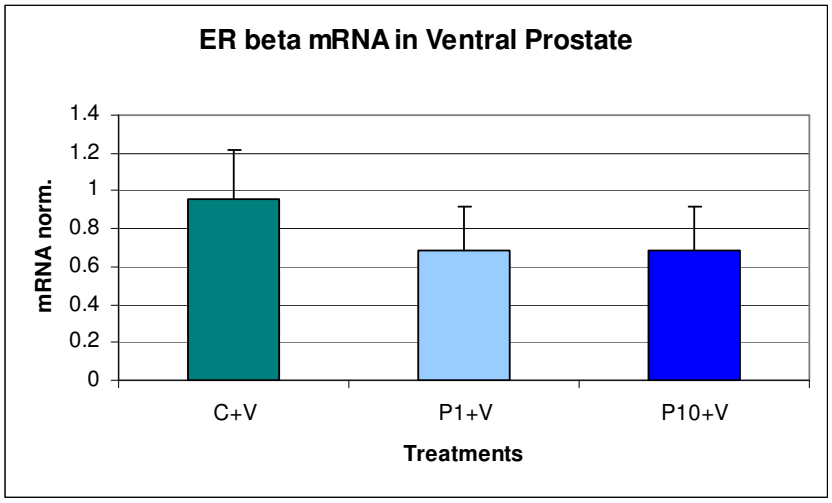
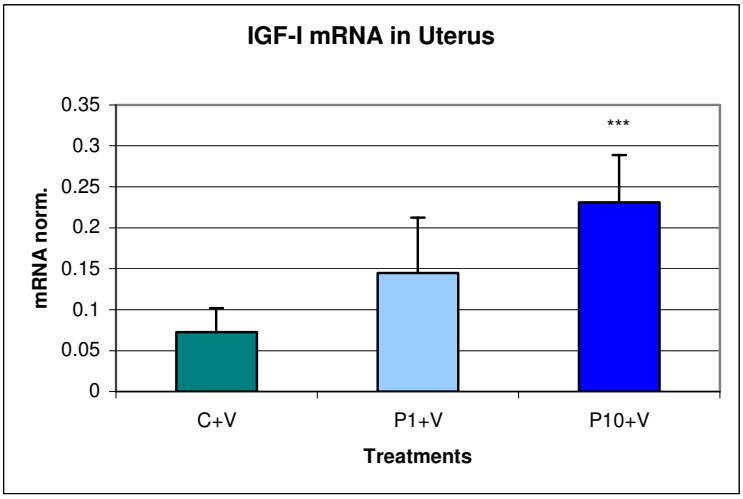


Figure 4.37: mRNA levels in Ventral Prostate of gonadectomized acute vehicle-injected rat offspring. mRNA amount was quantified by Real Time PCR and normalized to cyclophilin. Signicant differences are indicated by asterisks one*(p<0.05), two p<0.01) and three***(p<0.001).**

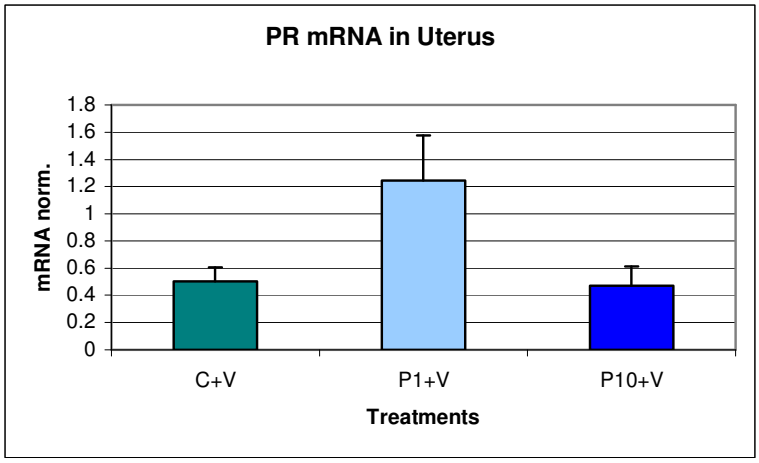
Ib Uterus

Uteri from gonadectomized female offspring at week 10 and exposed to vehicle (DMSO) at week 12, were analysed to determine the mRNA amount for IGF-I, PR, AR, ERα and ERβ. Significant differences as compared to vehicle-injected, gonadectomized controls were observed in IGF-I P10 group, ERβ P10 group and in AR P1 group (fig. 36). Comparing to the baseline levels there are not much similar tendencies.

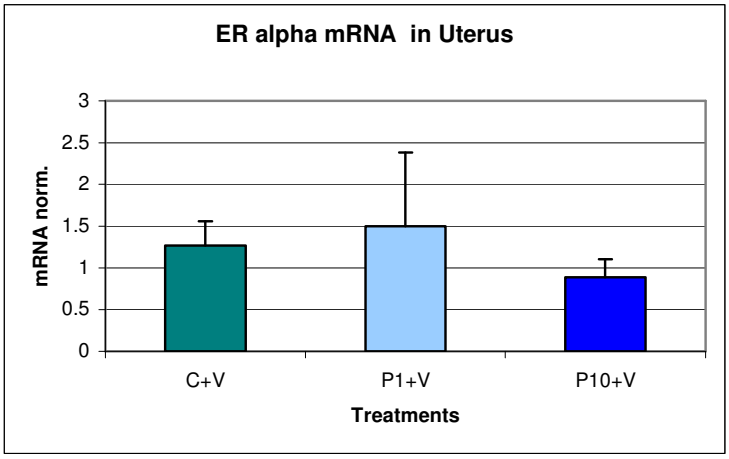
IGF-I mRNA levels in Uterus



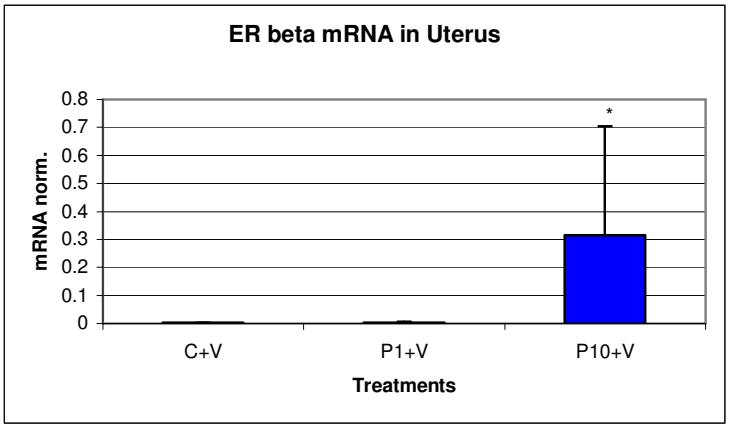
PR mRNA levels in Uterus



ER α mRNA levels in Uterus



ER β levels mRNA in Uterus



AR mRNA levels in Uterus

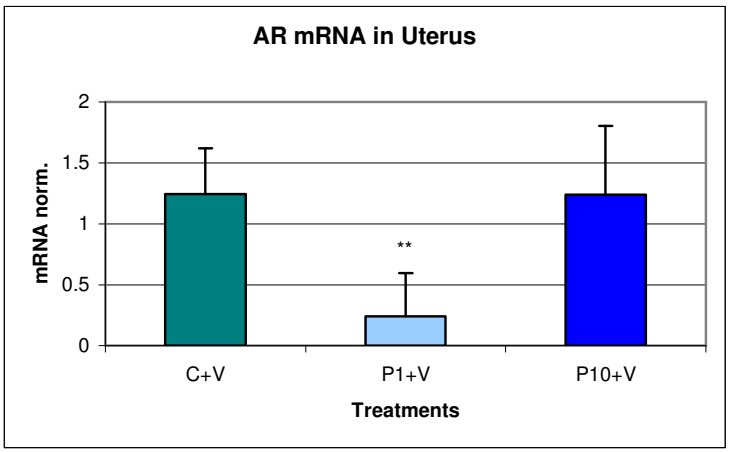


Figure 4.38: mRNA levels in Uterus of gonadectomized acute vehicle-injected rat offspring. mRNA amount was quantified by Real Time PCR and normalized to cyclophilin. Significant differences are indicated by asterisks one*(p<0.05), two p<0.01) and three***(p<0.001).**

Acute effect of estradiol in different chronic treatment groups

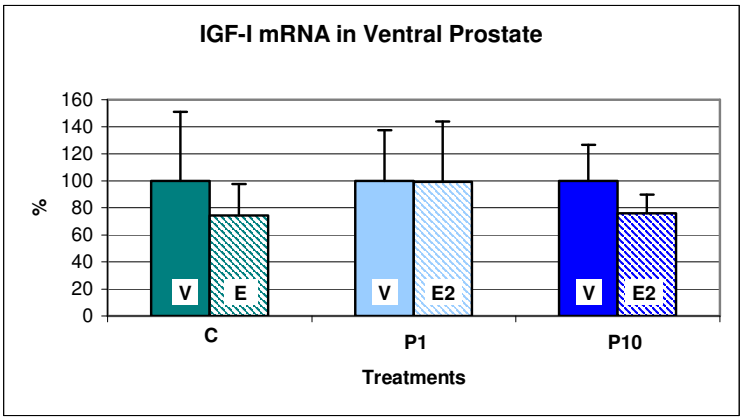
For every chronic treatment group, the mRNA levels of individual genes in acutely E2-injected rats were compared with those of rats injected with vehicle (fig.37). Levels were expressed as 100% of the corresponding vehicle-injected group.

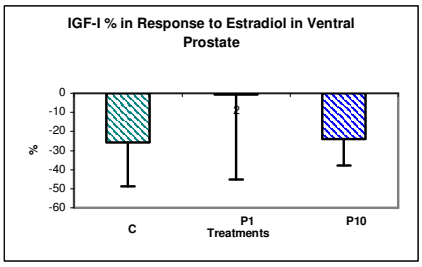
In **males** no significant estradiol-induced changes were observed except for AR in the P10 group (fig.37) which was significantly reduced by acute administration of estradiol ($p < 0.05$). Thus, the P10 group exhibited a response to Estradiol that was not seen in controls.

In **females**, the mRNA levels were significantly changed by E2 in almost all genes investigated (fig. 4.40). IGF-I (fig. 4.40) showed significant increases in all groups exposed to E2 compared to the vehicle group. PR (fig. 4.40) was significantly induced by E2 in P1 and P10, a similar tendency was also observed in controls. ERα was down- regulated in control and P1 groups (fig.4.39), with an analogous tendency in P10. The magnitude of the effect of Estradiol appeared to be dose-dependently reduced in the PBDE-exposed groups for IGF-I mRNA, and increased for ERβ mRNA. ERβ mRNA was quite difficult to detect by Real Time PCR. A significant down-regulation by E2 was noted in the P10 (fig. 4.40). The last gene investigated in uterus was AR (fig. 4.40) showing a down-regulation by E2 in the various treatment groups.

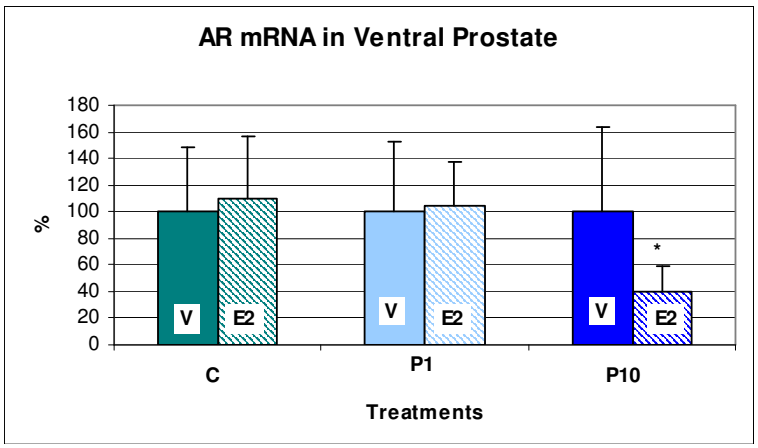
I a. Ventral Prostate

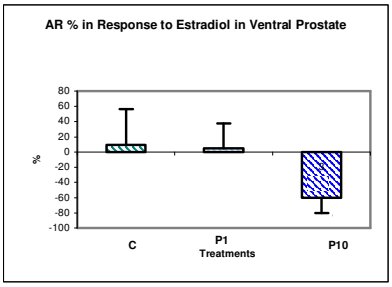
IGF-I mRNA levels in Ventral Prostate after E2 injection



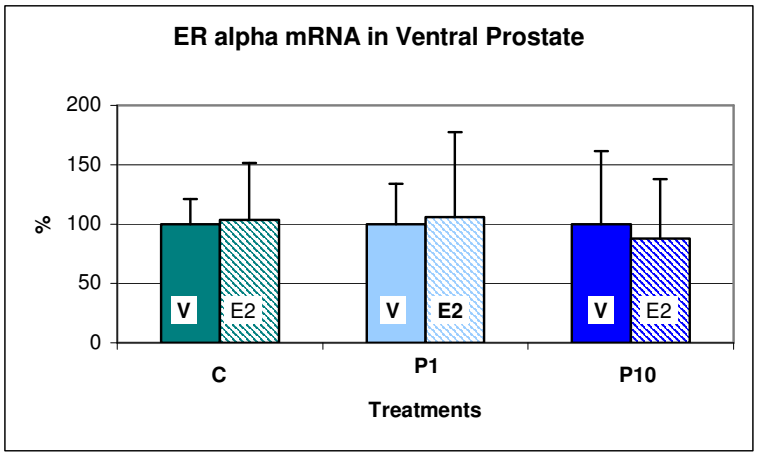


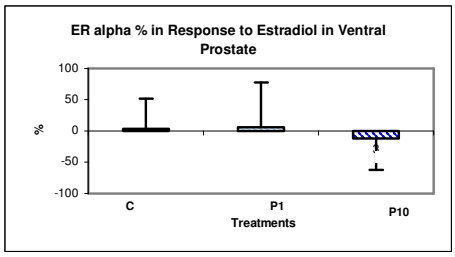
AR mRNA levels in Ventral Prostate after E2 injection



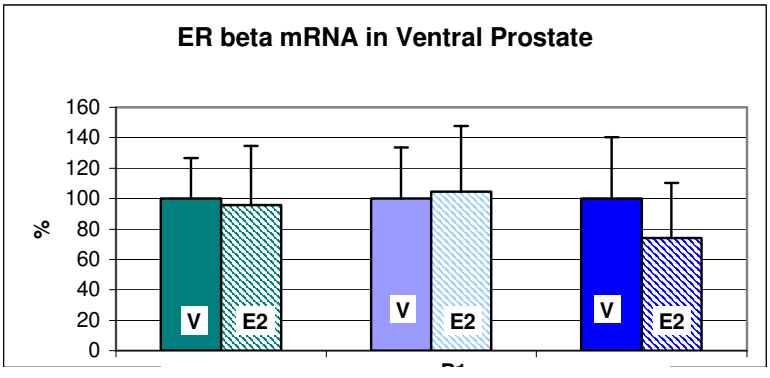


ERα mRNA levels in Ventral Prostate after E2 injection





ERβ mRNA levels in Ventral Prostate after E2 injection



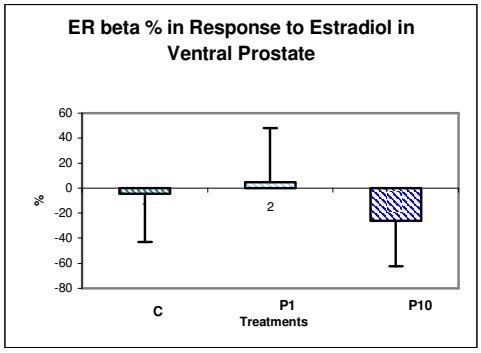
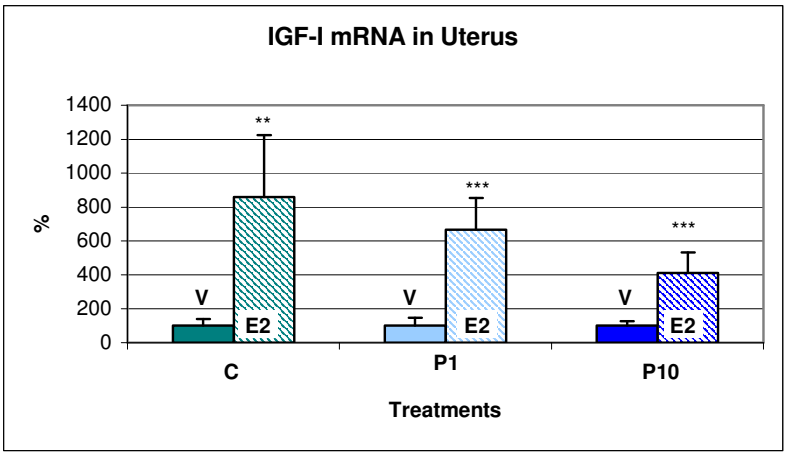
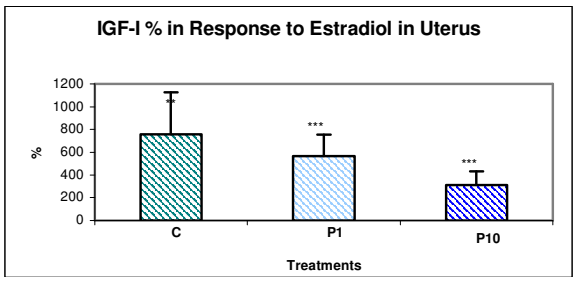


Figure 4.39: mRNA expression after acute injection of estradiol (10µg/kg s.c.) in ventral prostate of gonadectomized offspring. mRNA amount is expressed as percentage of the corresponding vehicle-injected control of the same chronic treatment group. DMSO was used as vehicle. mRNA levels for IGF-1, AR, ERα and ERβ were quantified by Real Time PCR and normalized to cyclophilin. Mean± S.D. Asterisk indicates p< 0.05 from corresponding vehicle-injected group.

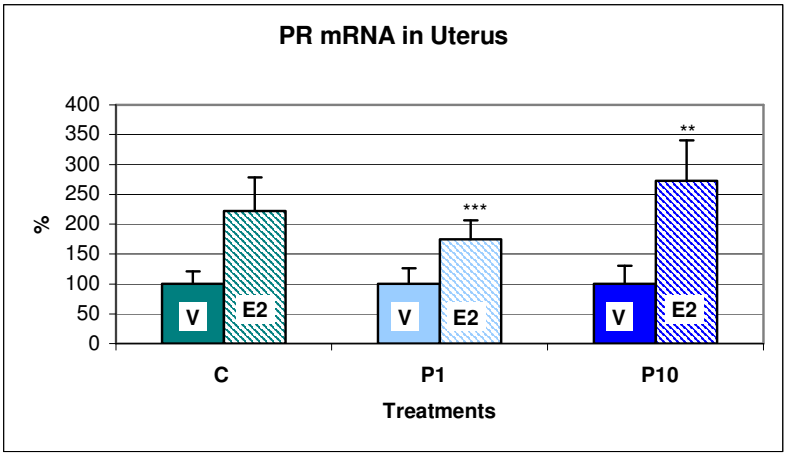
II b. Uterus

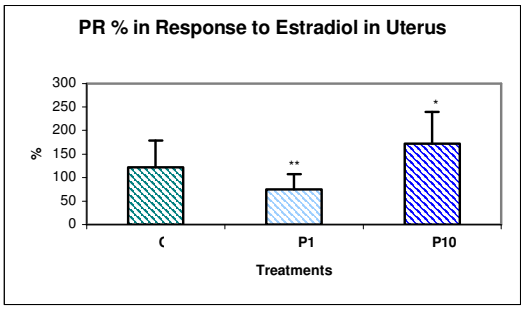
IGF-I mRNA levels in Uterus after E2 injection



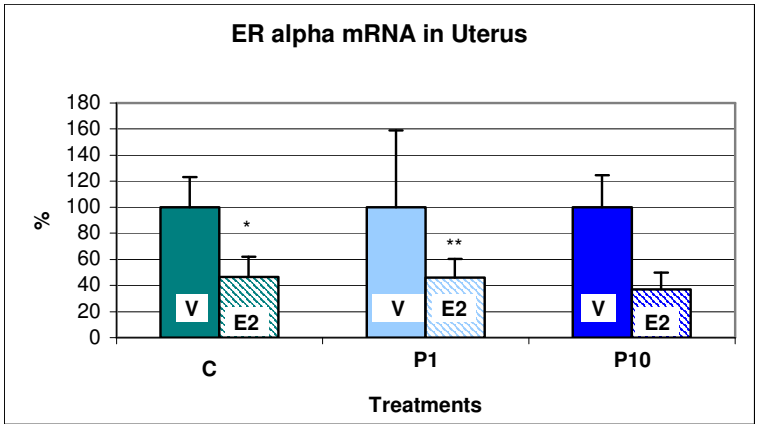


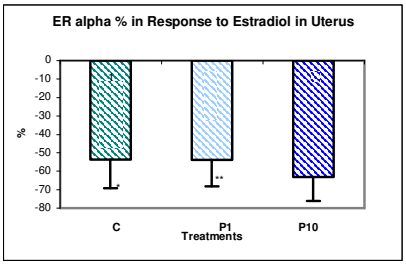
PR mRNA levels in Uterus after E2 injection



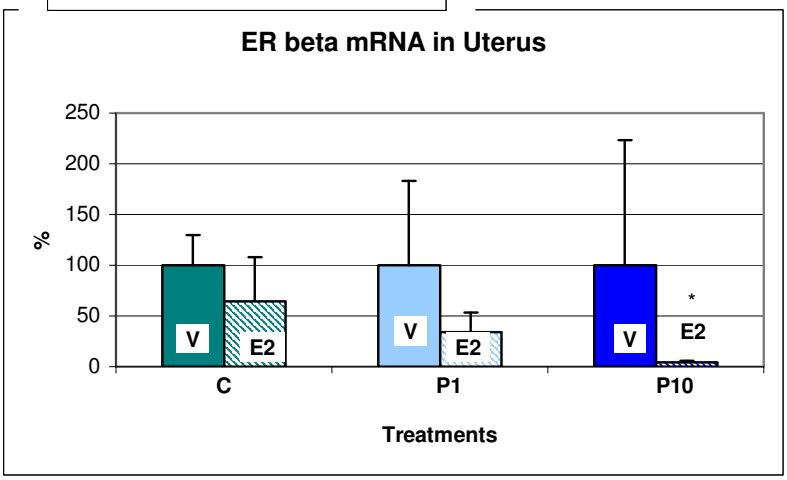


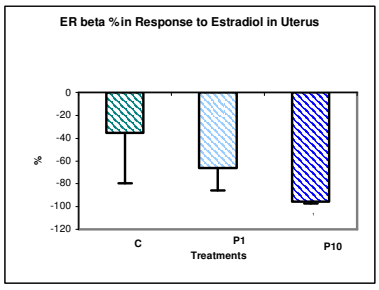
ERα mRNA levels in Uterus after E2 injection



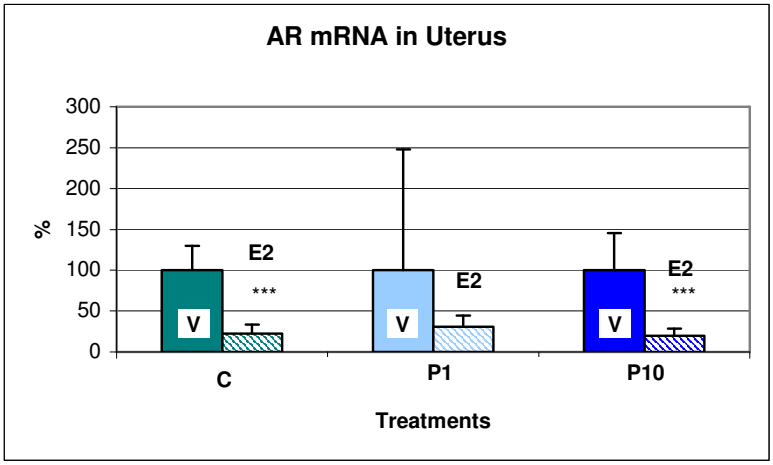


ERβ ...n





AR mRNA levels in Uterus after E2 injection



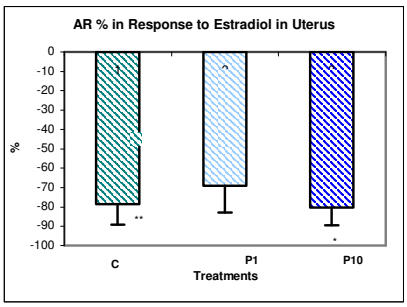


Figure 4.40: mRNA expression after acute injection of estradiol (10µg/kg s.c.) in uterus of gonadectomized offspring. mRNA amount is expressed as percentage of the corresponding vehicle-injected control of the same chronic treatment group. DMSO was used as vehicle. mRNA levels for IGF-I, PR, ERα and ERβ were quantified by Real Time PCR and normalized to cyclophilin. Mean± S.D. One asterisk indicates p< 0.05, two p< 0.01 and three p< 0.001 from corresponding vehicle-injected group.

4.7.2 Acute Estrogen Challenge Study with Olive Oil as Vehicle

The levels of estrogen target gene mRNAs after acute administration of E2 were also investigated using another kind of vehicle, the Olive oil. E2 was first dissolved in ethanol and then in sterileolive oil.

Olive oil with the same amount of ethanol was used as vehicle control. The genes investigated were IGF-I, PR, AR, ERα and ERβ in ventral prostate and uterus of Control, PBDE 99 1 mg/kg, PBDE 99 10 mg/kg and Aroclor 1254 10mg/kg treatment groups.

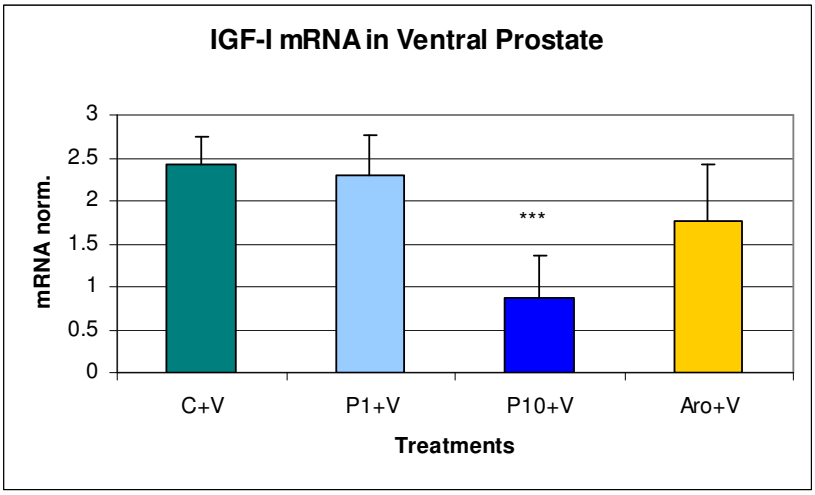
I. mRNA levels in acute vehicle-injected rats of different treatment groups

Ia Ventral Prostate

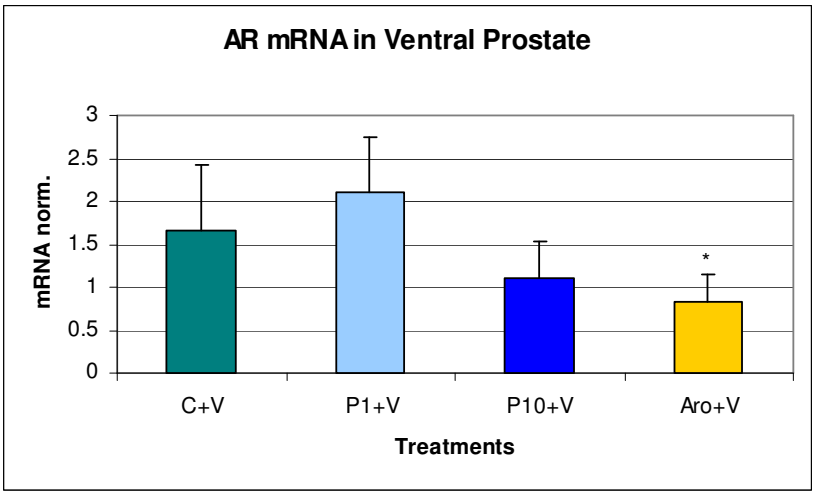
Ventral prostates of adult offspring gonadectomized at 10 weeks of age, sacrificed at week 12 and exposed to Vehicle (Olive oil,) were analysed for their mRNA levels of IGF-I, AR, ERα and ERβ.

A significant decrease, as compared to vehicle-injected controls was seen in IGF-1 mRNA levels in the P10 dose group. The Aro 10 group exhibited a decrease in AR and an increase in ERα mRNAs (fig. 4.41). In intact offspring studied under steady state conditions (fig. 4.25 and 4.28), the same tendency was seen in IGF-I and ER β although there were no significant changes.

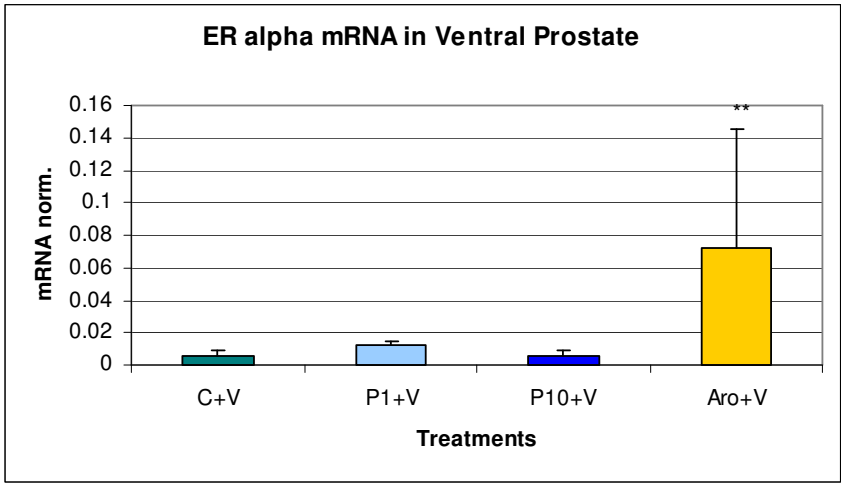
IGF-I mRNA levels in Ventral Prostate



AR mRNA levels in Ventral Prostate



ERα mRNA levels Ventral Prostate



ERβ mRNA levels in Ventral Prostate

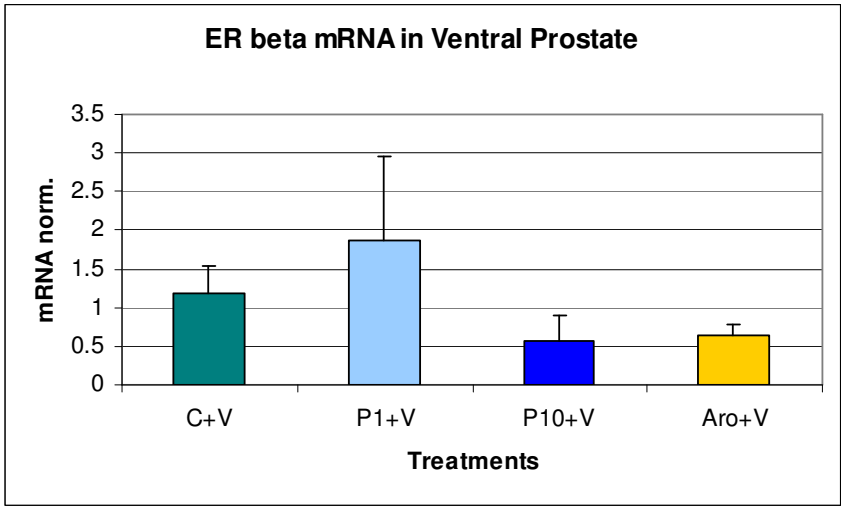
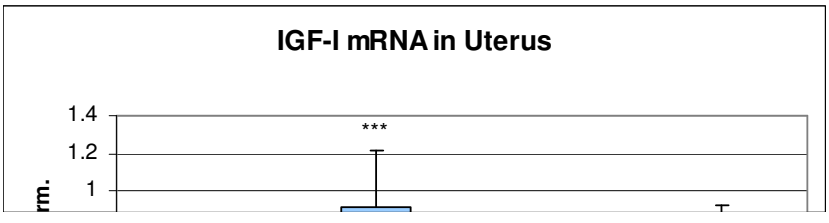


Figure 4.41: mRNA expression after acute injection of estradiol (10µg/kg s.c.) in ventral prostate of gonadectomized offspring. Olive oil was used as vehicle. mRNA levels for IGF-I, AR, ERα and ERβ were quantified by Real Time PCR and normalized to cyclophilin. Data represent Mean± S.D. Asterisk indicates p< 0.05 from corresponding vehicle-injected group.

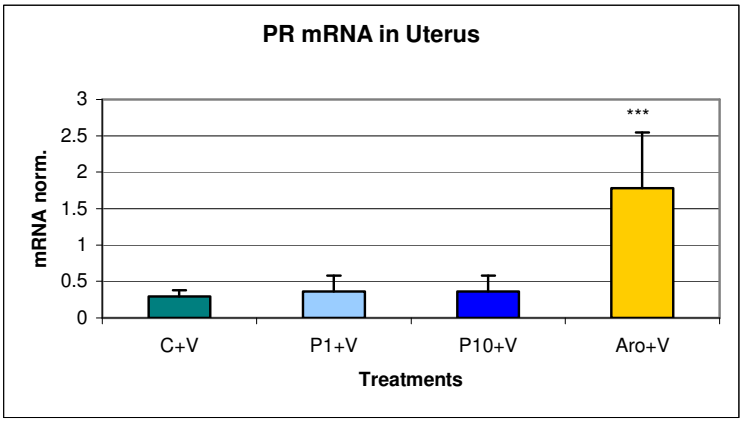
Ib Uterus

Uteri from gonadectomized female offspring at week 10 and exposed to vehicle (DMSO) at week 12, were analysed to determine the mRNA amount for IGF-I, PR, AR, ERα and ERβ. Significant changes were observed in IGF-I for P1 group, PR was above vehicle-injected controls in the Aro10 group, and ERα in the P1 group (fig. 40). In the steady state series there was similar tendency in IGF-I. ERα in the P1 group, there was a similar increase.

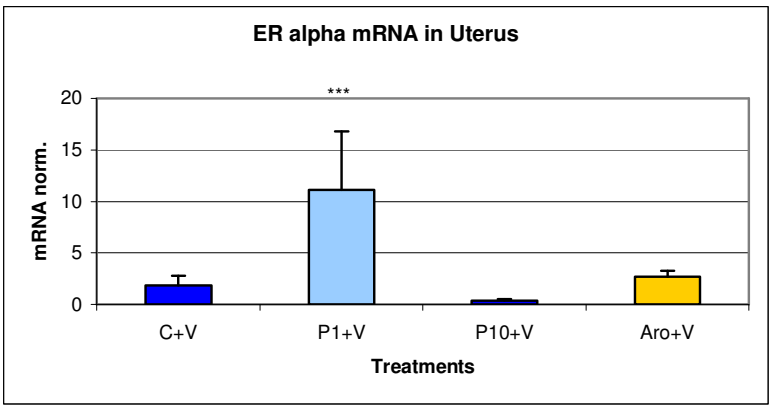
IGF-I mRNA in Uterus



PR mRNA in Uterus



ER α mRNA in Uterus



AR mRNA in Uterus

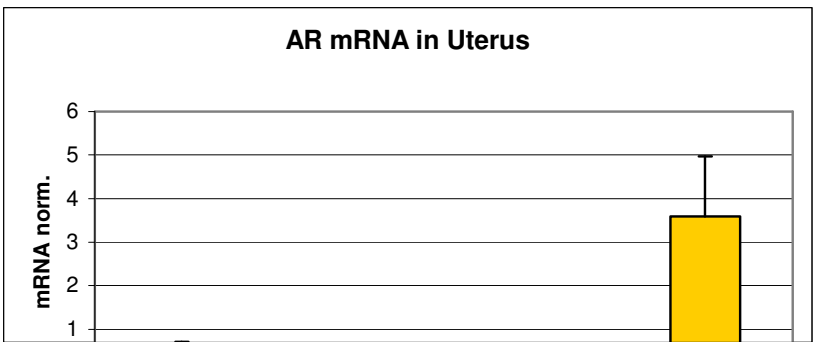


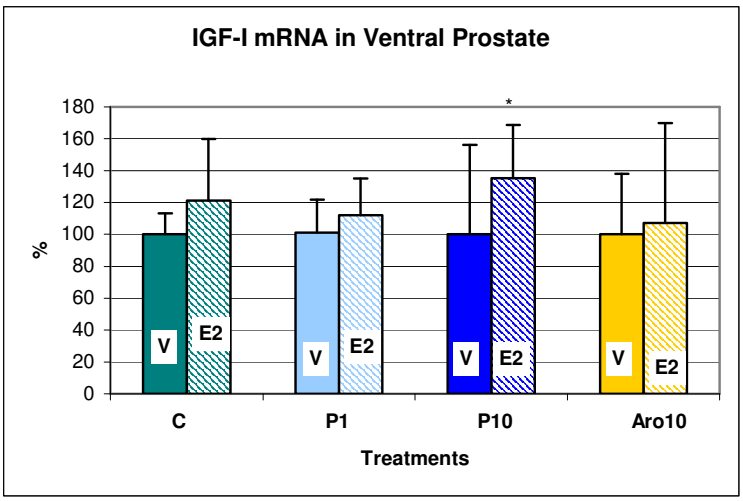
Figure 4.42: mRNA levels in gonadectomized animals and exposed to Vehicle. Olive oil was used as vehicle. The mRNA levels for IGF-I, PR, AR and ERα were quantified by Real Time PCR and normalized to cyclophilin. Data represent Mean± S.D. Asterisk indicates p< 0.05 illustrated with*, high significant changes (p<0.01) were indicated with ** and extremely significant (p< 0.001) with *.**

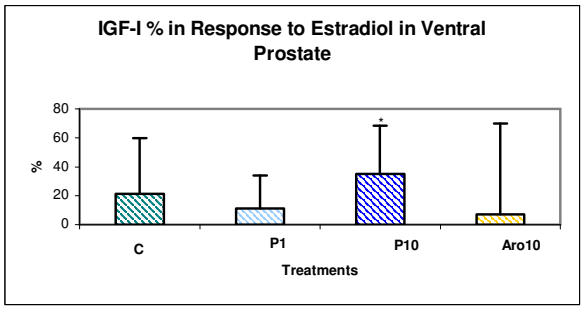
Acute effects of estradiol in different treatment groups

mRNA levels of estradiol-injected and vehicle-injected, gonadectomized rats were compared within every treated group, to see whether within the same group E2 influenced the mRNA level in ventral prostate and in uterus. Effects were expressed as percent of the vehicle-injected group to see eventual changes more clearly. In **males**, significant changes were observed for IGF-I in the P10 group (fig.41) which was significantly induced by E2 ($p< 0.05$). ERα was significantly elevated after E2 ($p< 0.001$) in P1 (fig.41).

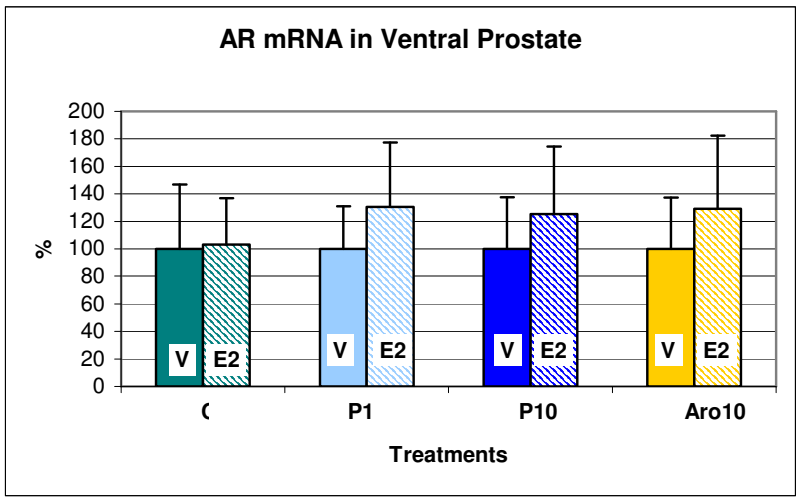
In **females**, the mRNA levels were significantly altered in almost all genes investigated for all treatment groups indicating high sensitivity to E2 stimulation (fig. 4.46). IGF-1 mRNA was induced by E2 in all treatment groups (fig. 4.44). PR mRNA was stimulated in P1 and Aro10. ERα was down-regulated in P1 (fig.4.46). AR mRNA quantification was very variable within the same treatment group which rendered analyses difficult. AR was significantly down-regulated in the Aro 10 group.

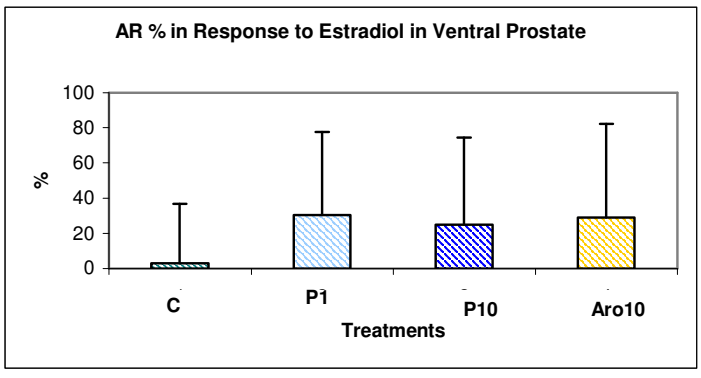
IGF-I mRNA levels in Ventral Prostate after E2 injection



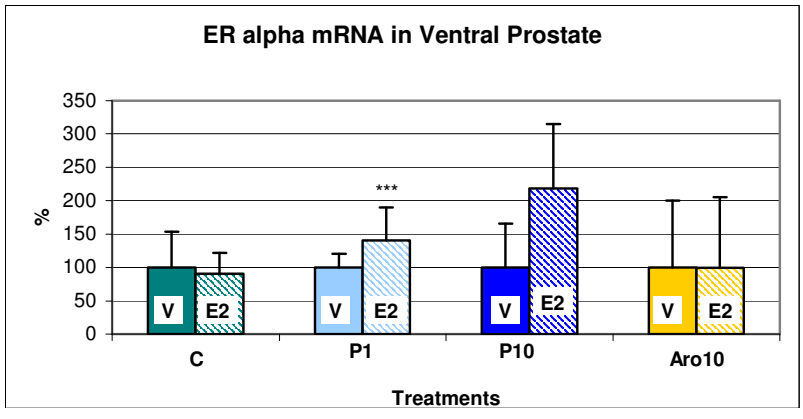


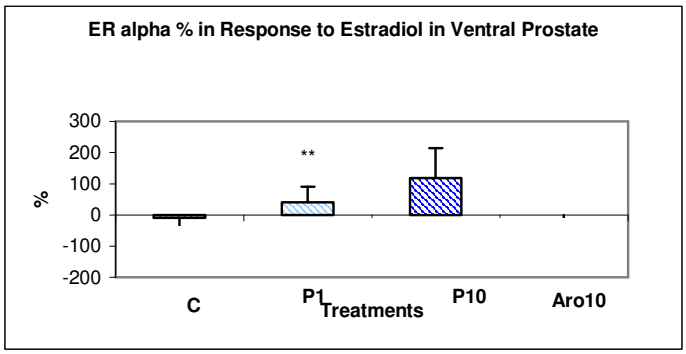
AR mRNA levels in Ventral Prostate after E2 injection



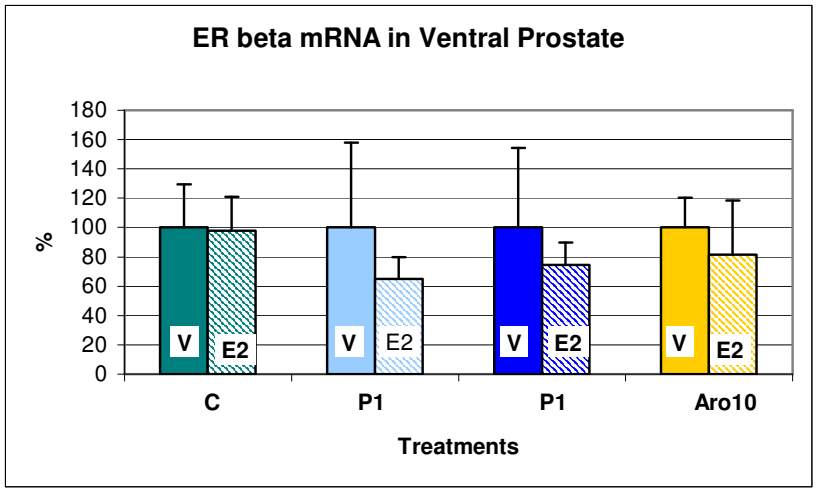


ERα mRNA levels in Ventral Prostate after E2 injection





ERβ mRNA levels in Ventral Prostate after E2 injection



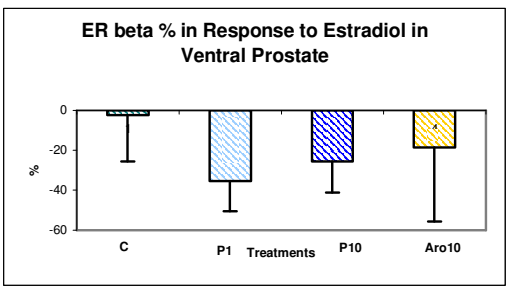
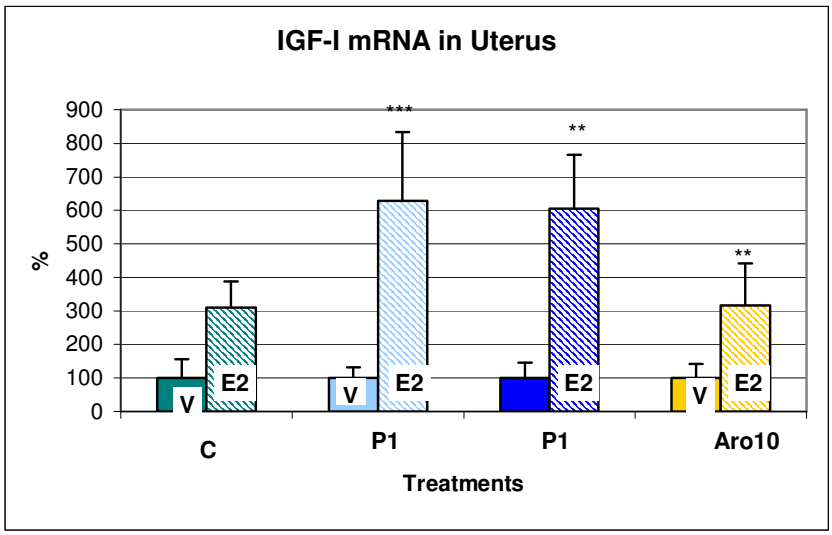
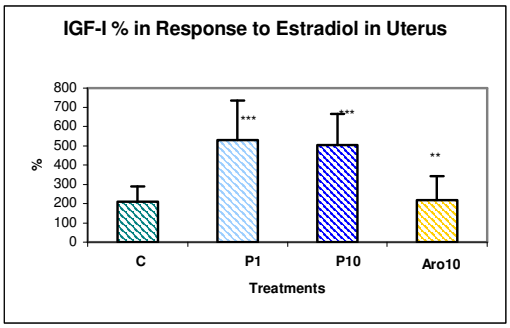


Figure 4.43: mRNA expression after acute injection of estradiol (10µg/kg s.c.) in ventral prostate of gonadectomized offspring as percentage of the corresponding vehicle-injected control of the same chronic

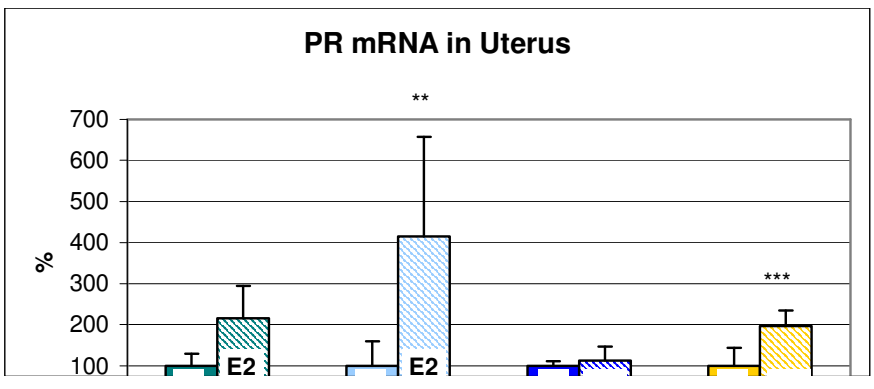
treatment group. Olive oil was used as vehicle. mRNA levels for IGF-I, AR, ERα and ERβ were quantified by Real Time PCR and normalized to cyclophilin. Mean± S.D. Asterisk indicates p< 0.05, two p< 0.001 from corresponding vehicle-injected group.**

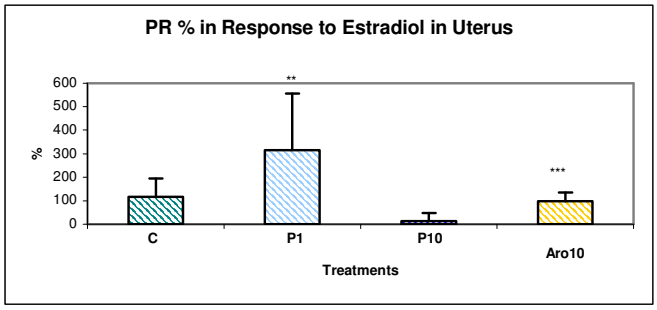
IGF-I mRNA in Uterus after E2 injection



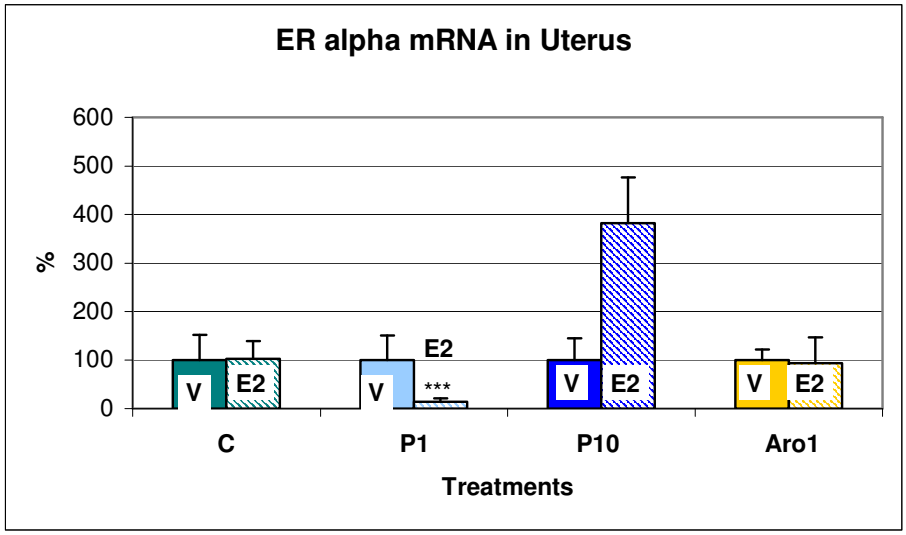


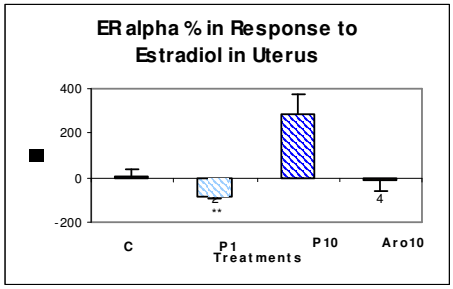
PR mRNA levels in Uterus after E2 injection





ERα mRNA levels in Uterus after E2 injection





AR mRNA levels in Uterus after E2 injection

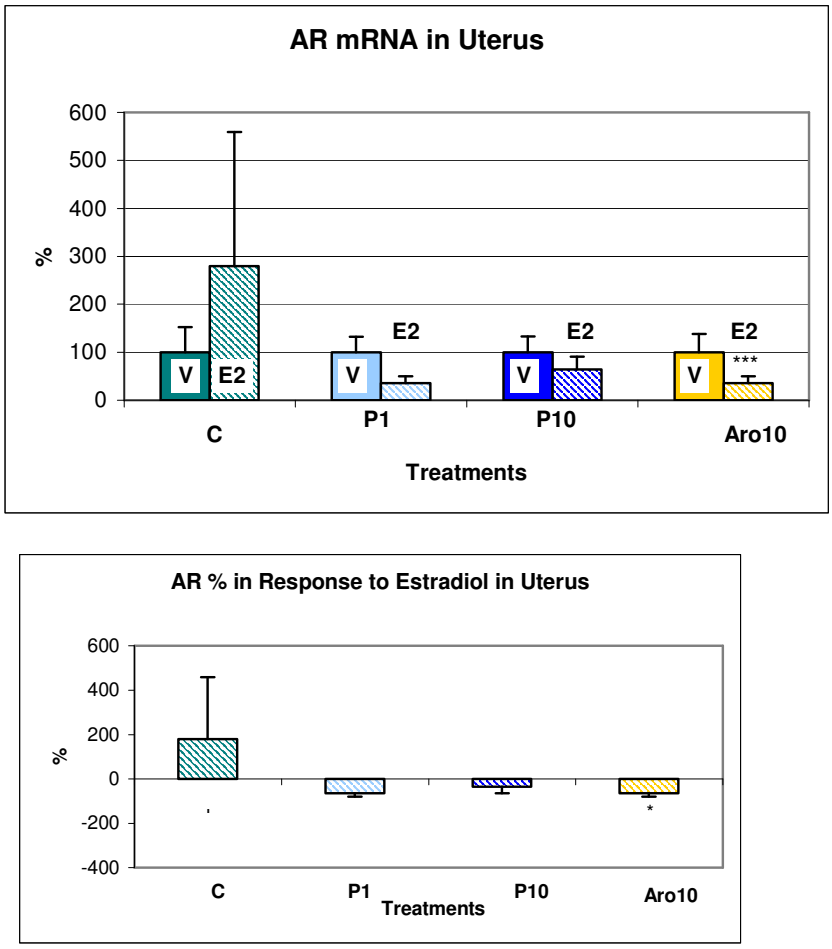


Figure 4.44: : mRNA expression after acute injection of estradiol (10µg/kg s.c.) in uterus of gonadectomized offspring. mRNA amount is expressed as percentage of the corresponding vehicle-injected control of the same chronic treatment group. Olive oil was used as vehicle. mRNA levels for IGF-I, AR, ERα and ERβ were quantified by Real Time PCR and normalized to cyclophilin. Mean± S.D. One asterisk indicates p< 0.05, two p< 0.01 and three p< 0.001 from corresponding vehicle-injected group.

5. Discussion

Polybrominated diphenylethers (PBDE) bioaccumulate which may influence the developing organism. The PCB mixture, Aroclor 1254 was chosen as positive control because of its structural similarity to PBDE 99. Depending on their structure, PCBs bind either to the aryl hydrocarbon receptor (a receptor able to bind dioxin-like compounds) or to estrogen receptors. Developmental effects of PCBs are known in experimental animals and humans, but, the mechanism of action remains largely unsolved. Although PBDE 99 and Aroclor 1254 have the same structure they may have different effects. What is thought to be common is their effects on thyroid and body weight, and neurotoxic effects after perinatal exposure. No information on prenatal effects on developing neuro-endocrine systems is currently available.

Prenatal exposure to PBDE 99 at the doses of 1mg/kg and 10 mg/kg supplied during gestation days 10 to 18, did not alter the survival rate and the body weight in adult offspring. These results suggest that PBDE99 does not exert a general toxic effect on development, but a more subtile action. This points to the need to further investigations.

5.1 Classical toxicological endpoints: Organ weights

Males

PBDE 99 and Aroclor caused small but significant changes in reproductive organ weights of adult offspring. This is relevant from the point of view of risk assessment, because it clearly demonstrates an adverse effect of these chemicals.

The low dose PBDE 99 had effects on epididymis with a reduction of weight. Relative weight was more affected. Most prominent is the increase in weight of the ventral and dorsal prostate after PBDE 99 (significant for absolute or relative weight depending on dose and tissue).

The dose-response relationship appeared to be bell shaped in ventral prostate, but normal in the dorsal lobe. Increased prostate weight has been observed after developmental exposure to estrogens or to the xenostrogen Bisphenol A (Saal et al., 1997; Nagel et al., 1997); however, PBDE 99 exhibits only very little, estrogenic activity in vitro (Meerts et al., 2001). Thus, the mechanism underlying the effect of PBDE on prostate weight remains uncertain.

Females

Adult uterine weight is not affected by prenatal PBDE 99 exposure with the two doses used, suggesting a lower sensitivity of uterus to PBDE. Relevant decreases of the uterine weight were observed in Aroclor 1254 exposed females.
It seems noteworthy that the PCB mixture, Aroclor, did not influence prostate weight. On the other hand, uterine weight was affected by Aroclor, but not by PBDE 99. This indicates differences in the effect patterns of PBDE and PCB.
The ovaries were not significantly affected when the absolute weight is considered, but with normalization to body weight, a significant increase was demonstrated in high dose PBDE 99 exposed females. An analogous effect was observed after 10 mg/kg Aroclor. This increase could be due to a stimulation of follicular numbers in ovaries.
In contrast to males, relative liver weight was increased in PBDE 99 exposed females (10 mg/kg). Whether this change could be due to effects on liver metabolism remains to be elucidated.

5.2 Gene expression in adult offspring under steady state conditions

Males
The gene expression analysis was carried out in males separately in ventral and dorsal prostate, since the lobes have different regulation patterns. It could not be assumed that PBDE 99 exposure would give the same effects in both lobes. Furthermore, the estrogen target gene regulation in rat prostate is still not fully understood. The two estrogen receptors (ER) undergo several changes in their expression in prostate gland during development from the newborn to adult. This change results from interactions of many factors (Ksano et al., 2003). External factors have the potential to interfere with some of these crucial mediators and may lead to an alteration of the expression pattern. In this case, we tested the capacity of compounds to exert a long-term effect.

Ventral Prostate

The genes investigated in ventral prostate showed several changes in their mRNA levels.
Both PBDE 99 doses affected the expression of mRNAs encoding for IGF-1, AR, ERα and ERβ.

IGF-I mRNA was decreased by both PBDE doses and also by Aroclor. A very marked decrease was detected in androgen receptor mRNA levels, where the reduction was almost six fold compared to control ventral prostate for both dosages. The ER β mRNA levels were reduced following a dose dependent manner. The ER α mRNA was difficult to detect in high dose exposed animals.

Aroclor 1254, at the dose level used, also affected these genes in ventral prostate (fig. 5.1).

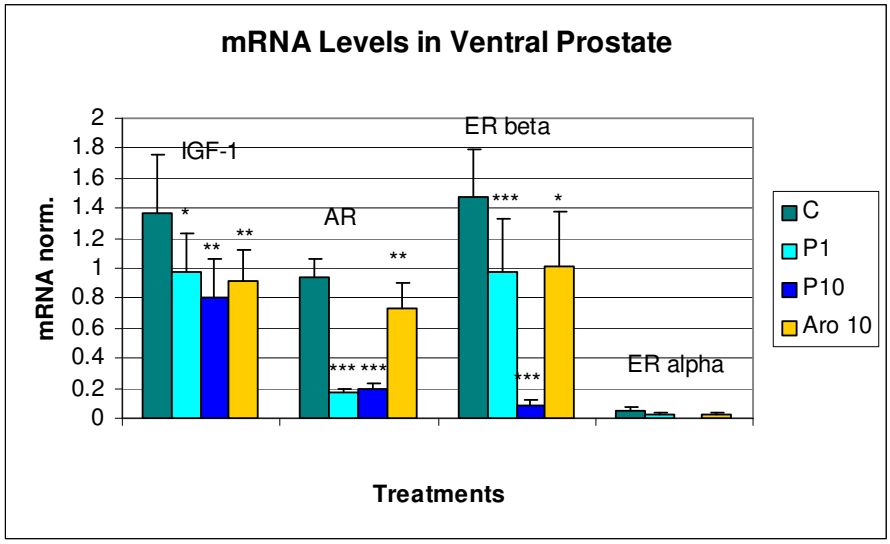


Figure 5.1: overview of estrogen target gene mRNA levels in ventral prostate. The quantification of IGF-I, AR, ERβ and ERα was done by Real Time PCR. The mRNA amount was normalized to cyc. A.

Thus estrogen target genes in ventral prostate are affected by prenatal exposure to PBDE 99 and Aroclor 1254 in a significant way, with a general decrease in mRNA levels, though the effect of the PCB mixture appear to be weaker than that of PBDE 99 (AR, ER β).

Several hypotheses could explain the mechanism by which PBDE down-regulates all the genes investigated. Since the adult rat offspring were still exposed to PBDE 99, due to its long half live, we cannot distinguish between effects resulting from prenatal exposure and effects due to the presence of PBDE 99 in adulthood. If the effects were due to the presence in the adult offspring, then PBDE 99 may inhibit the expression by antagonizing the effects of agonist mediators for IGF-I, AR and ER β, or alternatively by direct agonistic action. Finally, effects might also be mediated indirectly, e.g., on ER regulating AR and IGF-I. However, a reduction of ERβ would be expected to increase rather than decrease, AR. In uterus, (Weihua et al. 2001), it was shown that when ER β was eliminated, (for example in ER β knockout mice) AR was elevated, but in this case AR was decreased.

Dorsal Prostate

Dorsal prostate was also affected by PBDE 99 and Aroclor 1254. While the IGF-I mRNA expression was not altered by PBDE 99, the effect is different from what was observed in ventral lobe. AR mRNA levels were increased in a dose-dependent way. ER α was not changed in low dose exposure although the high dose elicited an increase by almost two fold. In ventral lobe, ER α was hardly detectable in the low dose and undetectable in the high dose group. ER β was down regulated to the same level by both PBDE 99 doses similar to the ventral lobe.

Exposure to Aroclor 1254 resulted in a prominent increase in IGF-I mRNA levels; the other genes did not seem to be altered by this compound (fig. 5.2). Again, the effect pattern was different from ventral lobe and from PBDE 99.

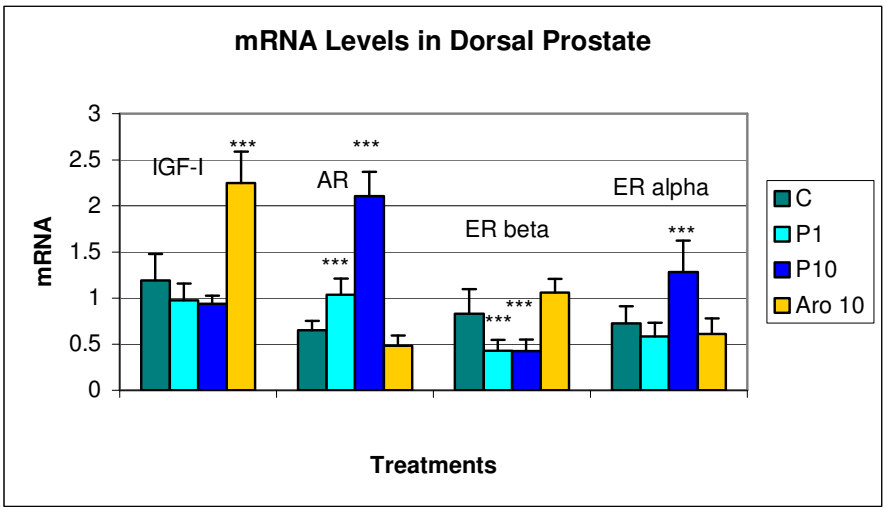


Figure 5.2: overview of estrogen target gene mRNA levels in dorsal prostate. The quantification of IGF-I, AR, ERβ and ERα was done by Real Time PCR. The mRNA amount was normalized to Cyc.

What comes out from these analyses is that PBDE 99 acts on rat prostate yielding different effects in the two lobes. Possible explanations could be that PBDE 99 has the same primary target but with opposite results due to different signalling mechanisms or that it acts on different targets. Dorsal and ventral prostate have lobe-specific secretory proteins, morphologic and functional differences (Hayward S.W. 2000) and may also have different gene expression patterns.

The dose-dependent increase in expression of AR mRNA after PBDE 99 exposure could be due to the decreased expression of ER β, after 10 mg/kg combined with up-regulation of ER α. Such a mechanism would be compatible with the one proposed by Weihua: ER β

appears to be a negative modulator of AR and acts to restrain the stimulatory action mediated by ER α (Weihua et al., 2000).

Alternatively, the change in mRNA levels may result from developmental effects. An increase in AR expression in mouse prostate linked with increased organ weight, has been observed after prenatal exposure to elevated levels of estrogen (Saal et al., 1997).

The discrepancy of the effects of PBDE 99 on gene expression in the two prostate lobes (strictly speaking three: ventral and dorsal + lateral) poses a problem for interpretation in terms of mechanism. Moreover, possible actions of PBDE 99 are difficult to predict because it is, at most, a very weak ER agonist (Meerts et al., 2001) which might exert antagonizing actions as a partial agonist. Some differentiation between prostate lobes is indicated by delayed effects of neonatal injection of estradiol. In adulthood, such rats showed a slight increase of ERα protein above control level in the lateral lobe (Prins et al., 1997) which is included in the "dorsal lobe" tissue pieces, whereas, ERβ was reduced in ventral prostate only (Prins et al., 1998) The balance between ERα and ERβ may, in turn, influence AR Expression.

Females

Uterus

Our results indicate changes in estrogen target gene mRNA levels in uterus of PBDE 99 exposed female rats. IGF-I mRNA level was not altered in a significant way in PBDE99 groups; this suggests that PBDE99 would not affect the rat uterine growth, as already seen in uterine weight data. Marked changes were observed in PR mRNA levels where there was a decline in a dose dependent manner (fig. 5.3).

ER α did not undergo significant changes. ER β mRNA exhibited a pronounced bell-shaped dose response curve with up-regulation in low dose and down-regulation in high dose exposed females (fig. 4). This response pattern does not appear to match that of classical estrogenic effects. Neonatal exposure to DES or coumestrol was found to lead to ERα down-regulation, while the effect on ERβ is not clear (Medloch et al., 1988; Khurama et al., 2000). Acute administration of ER agonists also down-regulates ERα and ERβ (Medloch et al., 1991); but, ERα returns to normal levels upon prolonged exposure (Sato et al., 1983). Uterine PR can be up- or down-regulated by estrogens depending on cell type (Weihua et al., 2000; Kurita et al., 2001a, 2001b); but, no change in PR mRNA levels was observed in our lab after prenatal DES exposure (Durrer, personal communication). Thus, the PR mRNA effect also appears to represent an atypical response pattern.

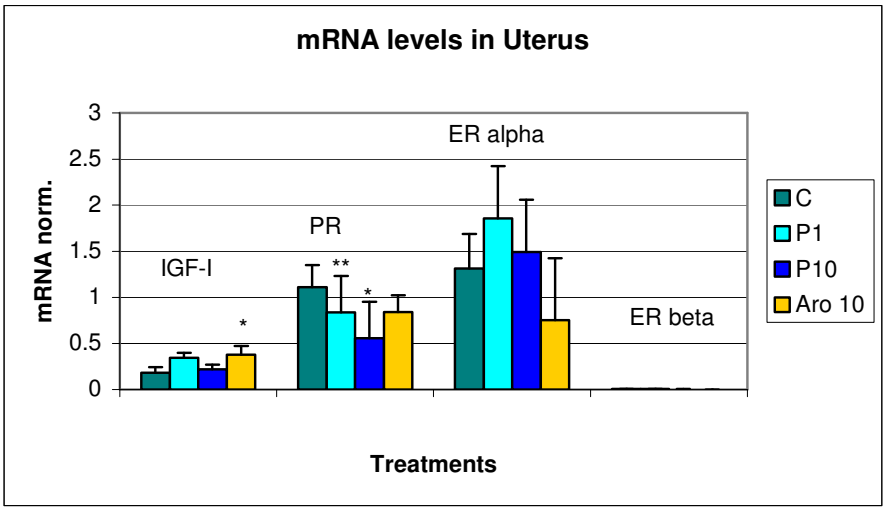


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Figure 5.3: overview of estrogen target gene mRNA levels in uterus. The quantification of IGF-I, PR, ERβ and ERα was done by Real Time PCR. The mRNA amount was normalized to cyc.

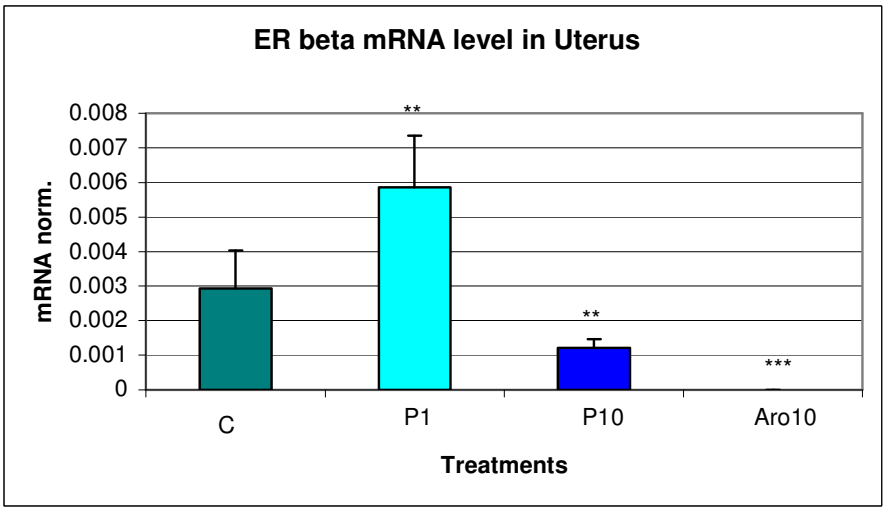


Figure 5.4: ER β mRNA level quantified by real time PCR in rat uterus. The mRNA amount was normalized to cyc. and measured in C, P1, P10 and Aro10 animals.

In previous investigations it was found out that ER α is a positive modulator for PR, and ER β is a negative modulator for ER α (Kurita et al., 2001)

Several mechanisms are being proposed, one is that ER β may form heterodimers with ER α inhibiting its inductive property on PR (Weihua et al., 2000).

PBDE 99 given at a dose of 1 mg/ kg induced ER β mRNA expression, which might explain the down regulation of PR. However, this explanation is not valid for the females

exposed to 10 mg/kg of PBDE 99, PR was strongly down-regulated in the presence of a marked down-regulation of ERβ and no change in ERα mRNA.

5.3. Acute Estrogen Challenge Study

Possible changes in the responsiveness of genes to estrogen were tested in 12 week old animals gonadectomized and exposed to 10 µg/kg 17β-Estradiol (E2), or to vehicle (DMSO). Two weeks later the acute effect of E2 was then checked 6 hours after E2 exposure. Gonadectomy has often been used to better understand gene regulation and the nature of action of estrogenic or anti-estrogenic compounds.

In males the response to E2 was analysed only in ventral prostate. Dorsal prostate is very reduced in size and difficult to dissect reliably after gonadectomy.

Adult offspring out of different litters of the various groups, C, P1 and P10 were exposed to E2 or vehicle (DMSO). Aroclor 1254 was not investigated because of insufficient number of offspring.

The mRNA amounts measured in gonadectomized animals were also compared to those measured in intact animals.

In gonadectomized **males** the mRNA levels in ventral prostate of treated groups did not differ much from the gonadectomized control group. Significant changes were observed in IGF-I, with a down-regulation in the P1 group, but levels were hold at the same level of the control in the P10 group.

When the pattern was compared to that in intact animals, it became evident that the type of effect of PBDE 99 differed:

AR and ERα mRNA levels in ventral prostate where not affected in gonadectomized animals in contrast to what was observed in intact offspring where there were marked decreases in both PBDE 99 treatment groups (fig. 5.5). ERβ tended to decrease in gonadectomized rats; but, the effect was not significant. In intact animals, there was a more prominent decrease after both PBDE 99 doses (fig. 5.5).

IGF-I mRNA showed a decrease in ventral prostate of both gonadectomized and intact animals, but in gonadectomized rats, the effect was restricted to the low dose group.

On the basis of these results, it is evident that the hormones produced by the testes and their action on receptors in ventral prostate play an important part in the manifestation of the PBDE effect pattern. The effect pattern appears to result from an interaction of (early and/or late) effects of the chemical with the action steroid hormones. One possible explanation would be that ERα, ERβ and AR in ventral prostate have an increased tendency to be down-regulated by circulating sex hormones.

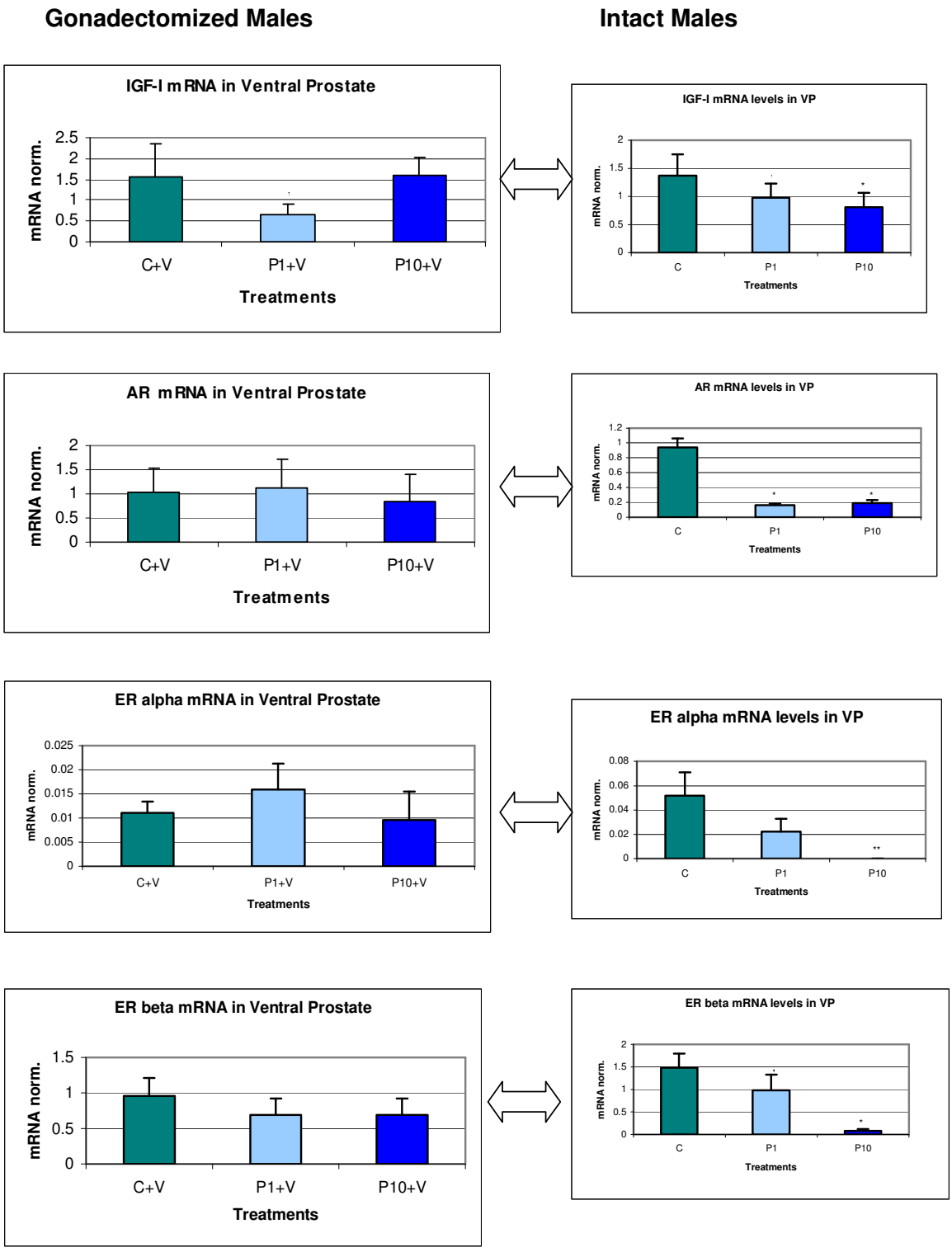
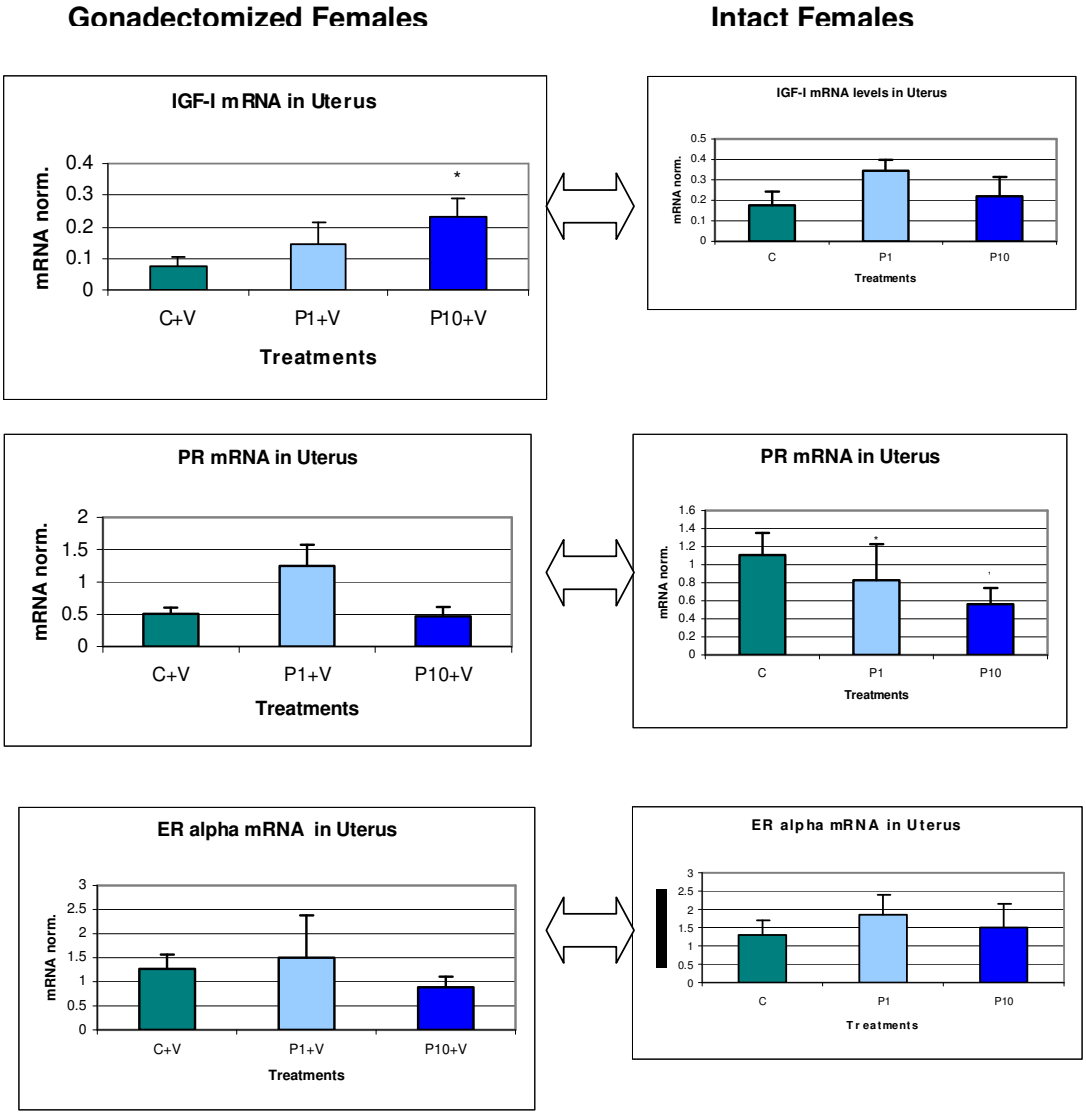


Figure 5.5: mRNA levels in ventral prostate of gonadectomized adult males (left) and intact adult animals (right). Control, PBDE99 1 mg/kg/day or PBDE99 10 mg/kg/day. The gonadectomized animals were injected with a Vehicle (DMSO). Quantification of IGF-I, AR, ERβ and ERα mRNA by Real Time PCR. mRNA amount of normalized to cyclophilin.

In **females,** mRNA levels where altered in ovariectomized offspring. IGF-I showed a dose dependent increase, ERβ showed an increase in PBDE 99 high dose group, and AR was down regulated in the low dose group. PR and ERα did not exhibit significant changes.
When we compare the mRNA levels of gonadectomized females to those of intact ones, the effect patterns of PBDE 99 again differ in part, in analogy to males. ERβ differs completely between gonadectomized and intact animals. IGF-I has in common an increase in P1 group, but shows a bell-shaped dose-response in intact animals and a monotonic one in gonadectomized males. A shift from monotonic to bell-shaped dose-response is noted for PR mRNA. AR was not measured in baseline animals (fig. 5.6).
Thus, there also appear to exist an interaction of PBDE 99 with hormones secreted by the ovaries.



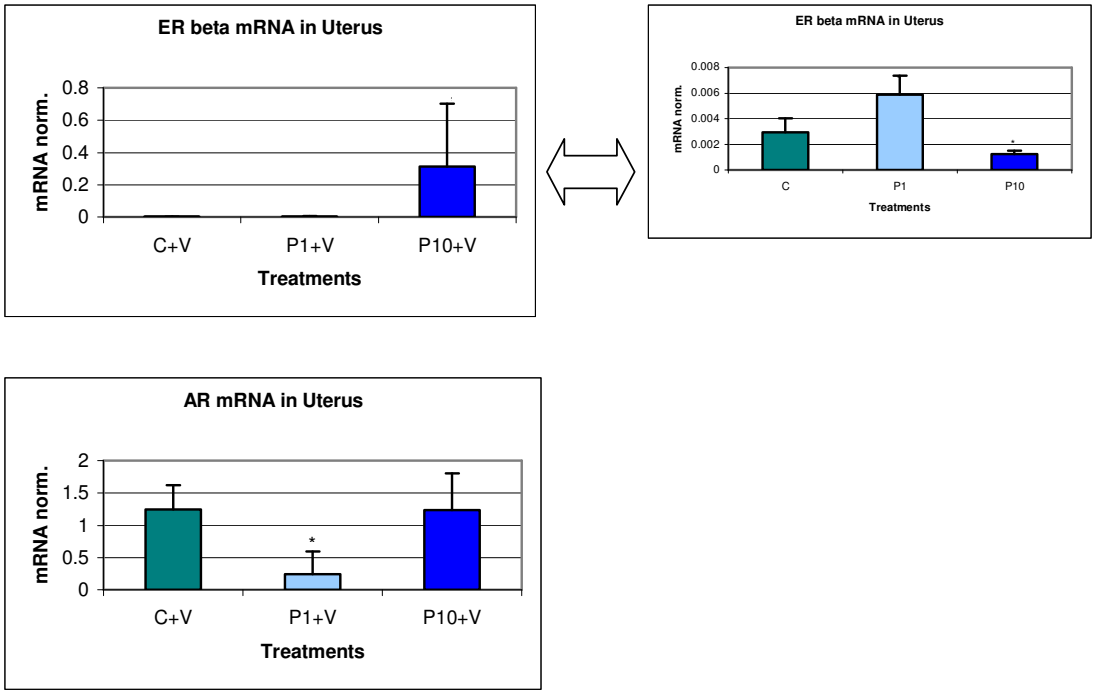


Figure 5.6: mRNA levels in uterus of gonadectomized adult females (left) and intact adult animals (right). Control, PBDE99 I mg/kg/day or PBDE99 10 mg/kg/day. The gonadectomized animals were injected with a Vehicle (DMSO). Quantification of IGF-I, PR, ERβ, ERα AR mRNA by Real Time PCR. mRNA amount of normalized to cyclophilin.

Acute response to estradiol in the various treatment groups

Males

Estrogen sensitivity of genes was assessed as percentage change in the various treatment groups.

IGF-I mRNA levels in the control group, decreased in response to E2, as compared to the control group injected with vehicle. In contrast, no change was detected in the P1 group. In the P10 group, there was a slight estrogen-induced decrease (fig. 5.7).

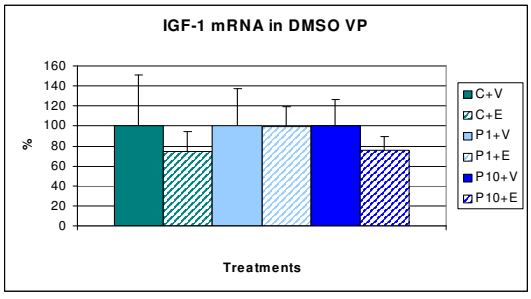


Figure 5.7: IGF-I mRNA expression in ventral prostate of gonadectomiezed rats. IGF-1 mRNA levels in vehicle (DMSO) (V) and estradiol (E2) groups. Data are expressed as % of the respective vehicle-injected group of the same treatment group.

AR mRNA, was down-regulated by the dose of E2 used (10 µg/kg) only in P10 gonadectomized males, it might be that PBDE 99 enhances down-regulation of AR mRNA by E2.

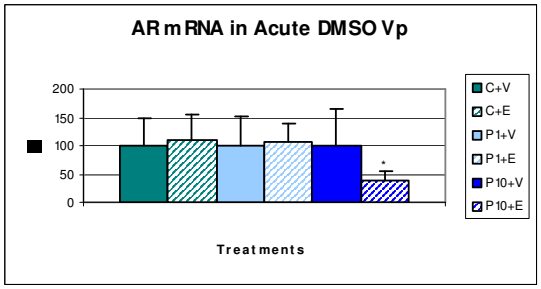


Figure 5.8: AR mRNA expression in ventral prostate of gonadectomiezed rats. AR mRNA levels in vehicle (DMSO) (V) and estradiol (E2) groups. Data are expressed as % of the respective vehicle-injected group of the same treatment group.

ER α and **ERβ** mRNA levels, were not significantly influenced by the acute E2 treatment. Subtile effects may have been obscured by the comparatively high variability in the groups. Thus, it was difficult to analyse estrogen sensitivity in this tissue in animals of the present experimental series. A more extensive dose range of E2 would have been necessary. The data with IGF-I suggest that low-dose exposure to PBDE 99 increased the sensitivity of this gene to E2 in ventral prostate.

From these results it seems that males are not highly sensitive to exposure to 17β-estradiol.

In **female** uterus, the estrogen target gene mRNA levels exhibited marked changes 6 hours after E2 (10 µg/kg).

An acute increase in **IGF-I** mRNA levels is in agreement with the known up-regulation of this gene by estrogen (Norstedt et al., 1989; Klotz et al., 2000). If we compare each estradiol group with the corresponding vehicle, we realize that induction is reduced in the PBDE 99 exposed groups in a dose dependent manner (fig. 5.9).

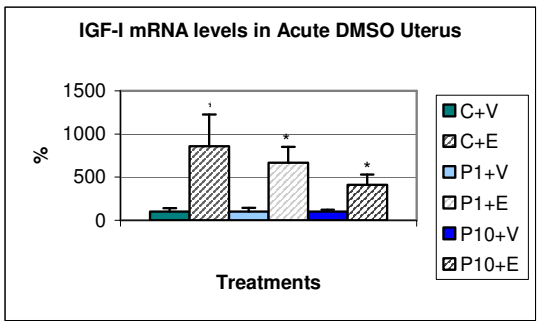


Figure 5.9: IGF-I mRNA expression in uterus of gonadectomized rats. IGF-I mRNA levels in vehicle (DMSO) (V) and estradiol (E2) groups. Data are expressed as % of the respective vehicle-injected group of the same treatment group.

As mentioned above**, PR** in uterus can be up- or down-regulated by estrogen depending upon tissue (Medlock et al., 1988; Khurama et al., 2000). It appears from the data in the control group that the stimulatory effect dominated in our sample. The induction of PR mRNA by E2 was enhanced in the PBDE 99-exposed rats in a dose dependent way (fig. 5.10).

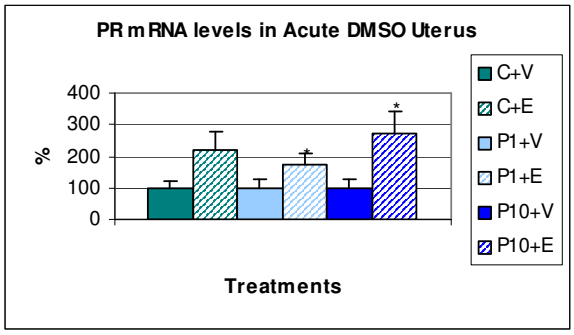


Figure 5.10: PR mRNA expression in uterus of gonadectomized rats. PR mRNA levels in vehicle (DMSO) (V) and estradiol (E2) groups. Data are expressed as % of the respective vehicle-injected group of the same treatment group.

The down-regulation of **ER α** mRNA levels (Medlock et al., 1999) in uterus was similar in Control and PBDE 99-exposed groups.

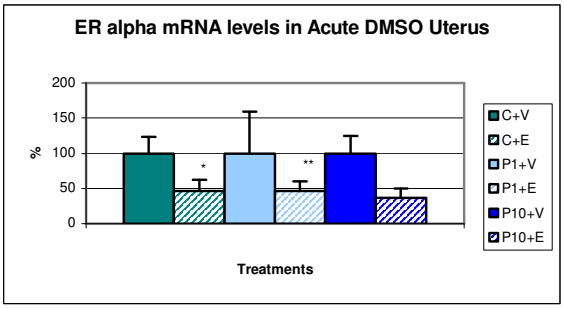


Figure 5.10: ER α mRNA expression in uterus of gonadectomized rats. ER α mRNA levels in vehicle (DMSO) (V) and estradiol (E2) groups. Data are expressed as % of the respective vehicle-injected group of the same treatment group.

In contrast, the repressive effect of E2 on uterine **ER β** mRNA levels appeared to be dose-dependently enhanced in PBDE 99-exposed animals.

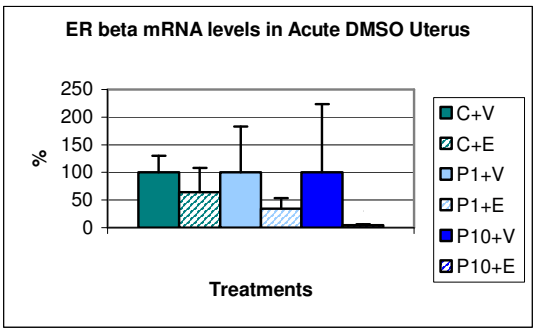


Figure 5.11: ER β mRNA expression in uterus of gonadectomized rats. ER β mRNA levels in vehicle (DMSO) (V) and estradiol (E2) groups. Data are expressed as % of the respective vehicle-injected group of the same treatment group.

AR mRNA levels are also down-regulated by E2 in uterus (Diel et al., 2000; Waters et al., 2001). This effect was not markedly affected by PBDE 99 exposure.

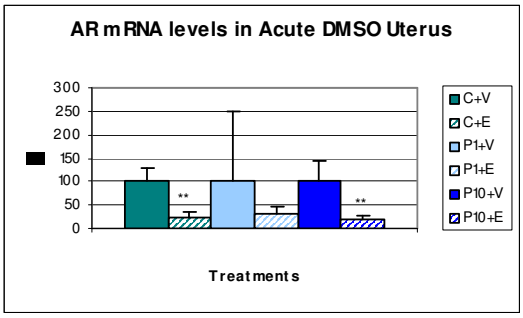


Figure 5.12: AR mRNA expression in uterus of gonadectomized rats. AR mRNA levels in vehicle (DMSO) (V) and estradiol (E2) groups. Data are expressed as % of the respective vehicle-injected group of the same treatment group.

Taken together, these data indicate gene-specific changes in estrogen sensitivity in uterus, reduction with IGF-I and enhancement with PR and ERβ. In ventral prostate, the response of IGF-I to E2 was also reduced by PBDE 99 (1 mg/kg).

Effect of Estradiol with olive oil as vehicle

Similar experiment was carried out on gonadectomized animals using olive oil as vehicle. This experiment also included animals exposed to 10 mg/kg Aroclor 1254.
In ventral prostate of **male** controls**, IGF-I** mRNA levels were not altered by E2 injected in olive oil (fig. 5.13). PBDE-exposed groups exhibited a tendency towards an increase of levels after E2, but was not significant.

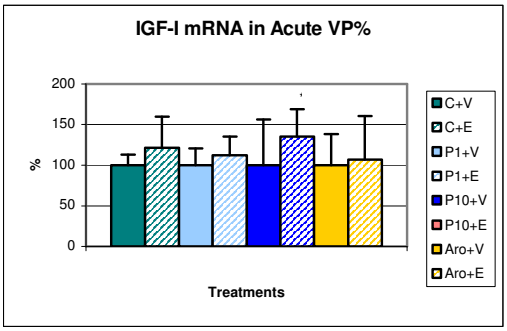


Figure 5.13: IGF-I mRNA expression in ventral prostate of gonadectomized rats injected with Vehicle (olive oil) or E2 in olive oil. Data are expressed as % of the respective vehicle group.

AR mRNA was not altered in controls. Levels in PBDE 99- and Aroclor-exposed groups again tended to increase after estradiol injection; but, the change did not reach statistical significance control (fig. 5.14).

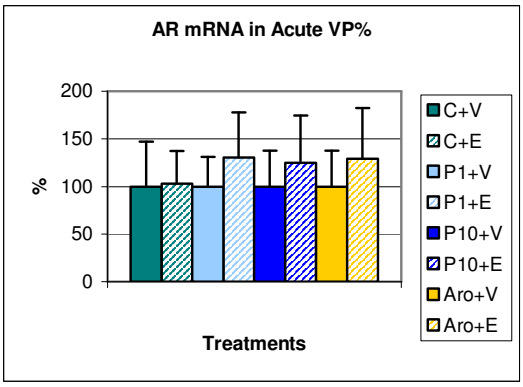


Figure 5.14: AR mRNA expression in ventral prostate of gonadectomized rats injected with Vehicle (olive oil) or E2 in olive oil. Data are expressed as % of the respective vehicle group

ER α mRNA levels in ventral prostate were unaffected by E2 in controls, but increased by E2 in both PBDE 99 groups with a significant difference from vehicle control after 1 mg/kg in PBDE 99 (fig. 5.15).

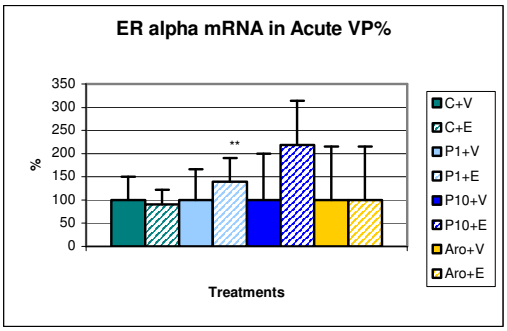


Figure 5.15: ER α mRNA expression in ventral prostate of gonadectomized rats injected with Vehicle (olive oil) or E2 in olive oil. Data are expressed as % of the respective vehicle group

ER β mRNA levels were not altered in Controls. The slight reduction by E2 in PBDE 99 treated animals was not significant (fig. 5.16).

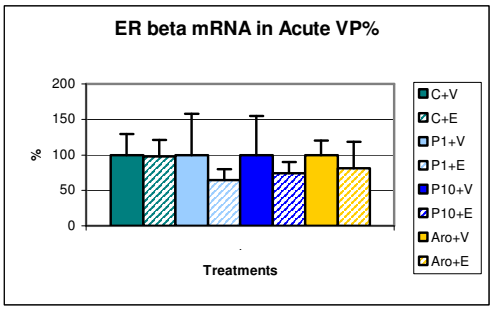


Figure 5.16: ER β mRNA expression in ventral prostate of gonadectomized rats injected with Vehicle (olive oil) or E2 in olive oil. Data are expressed as % of the respective vehicle group

In summary, there appeared to exist a difference between the effect of E2 in the case of IGF-I mRNA which was down-regulated by E2 in DMSO, but not affected by E2 in olive oil

injected in controls and for ERα after PBDE 99. Since changes in the remaining mRNAs did not reach statistical difference, a comparison of the two treatments is not possible.

In **females**, IGF-I, PR, ERα and AR mRNAs were investigated in uterus. **IGF-I** mRNA levels were increased much less in response to E2 in olive oil (300%) than in response to E2 in DMSO (800%). Surprisingly, the induction by E2 was enhanced in PBDE 99-exposed groups in comparison with the very low stimulatory level in controls. The effect remained below the level reached in controls in the DMSO-experiment (600 vs 800%), and hence, is difficult to interpret.

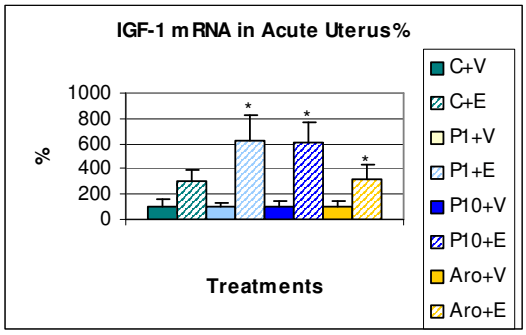


Figure 5.17: IGF-I mRNA expression in uterus of gonadectomized rats injected with Vehicle (olive oil) or E2 in olive oil. Data are expressed as % of the respective vehicle group.

PR mRNA in uterus was induced by E2 injection similar to the experiment with DMSO. Aroclor 1254-exposed rats also responded to E2. Apparent differences in the magnitude of the response to E2 did not reach statistical significance (fig. 5.18).

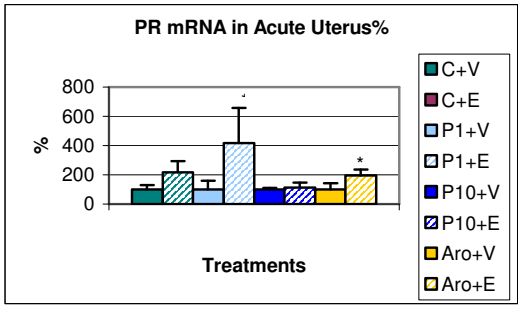


Figure 5.18: PR mRNA expression in uterus of gonadectomized rats injected with Vehicle (olive oil) or E2 in olive oil. Data are expressed as % of the respective vehicle group.

Surprisingly **ER α** was not down-regulated by E2 (in olive oil) in controls. The P1 exhibited down-regulation (similar to the DMSO experiment); whereas, the P10 group showed an opposite effect (fig. 5.19).

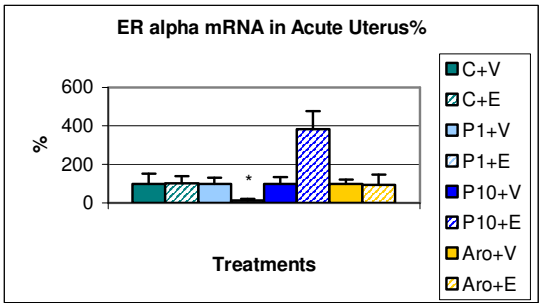


Figure 5.19: ER α mRNA expression in uterus of gonadectomized rats injected with Vehicle (olive oil) or E2 in olive oil. Data are expressed as % of the respective vehicle group.

AR mRNA levels were not significantly affected by E2 given in olive oil in controls and PBDE 99-exposed groups (with exception of Aroclor exposed animals which exhibited down-regulation) (fig. 5.20). A similar tendency was noticed in the P1 group.

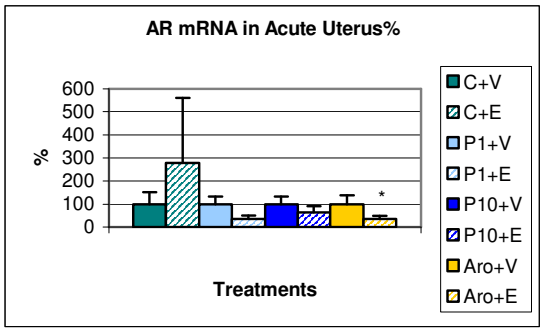


Figure 5.20: AR mRNA expression in uterus of gonadectomized rats injected with Vehicle (olive oil) or E2 in olive oil. Data are expressed as % of the respective vehicle group.

These data again contrast with those of the DMSO experiment where a significant down-regulation of AR mRNA levels was observed also in controls.

While the expected induction of IGF-I and PR could be demonstrated also with E2 given in olive oil (though with a smaller effect in case of IGF-I mRNA), the failure to detect acute down-regulation of ERα and AR mRNA with E2 dissolved in olive oil renders this experiment rather uncertain. The E2/DMSO data of controls fit better with data from the literature. Thus, DMSO seems to be better suited as vehicle for this purpose at least with respect to responses of the two organs studied. It is not clear what might be the difference between the two vehicles. It is possible that absorption might be slower from the subcutaneous olive oil depot. Since the acute response of the controls to E2 is difficult to judge, the changes seem in PBDE 99- and Aroclor 1254- exposed animals remain uncertain.

6. General discussion

Since PBDE 99 was identified in human samples as one of the main PBDE congeners, it is important to understand its effects. Concern about PBDEs arose when it was realized that concentrations started to rise exponentially, indicating a potential to be accumulated.
In contrast to the well-known PCBs mechanisms, the effects of PBDE are mostly unknown. Our main focus was to investigate whether PBDE 99 has effects on development, whether it acts on developing neuroendocrine systems, and whether the actions corresponded to affect of endocrine disruptors.
In rat offspring exposed to PBDE 99 during development and studied under steady state conditions, several changes in reproductive organ weights were detected in both sexes. They were accompanied by changes in estrogen target gene expression patterns. This means that PBDE 99 can affect developmental processes in reproductive organs. Since the chemical was still present in adult offspring, it cannot be determined whether the observed changes were a result of effects during early ontogeny or at later stages or, most probably, of a combination of both. It is also important to note that the dose-response relationship of PBDE 99 was not always monotonic, but in some cases appeared to be bell-shaped, as far as can be judged from two doses. This phenomenon is often observed with endocrine active chemicals. Finally, it is evident that the PCB mixture, Aroclor 1254, caused a different effect pattern, in spite of structural similarities between PBDE and PCB.
The effect of PBDE 99 on the sensitivity of genes to estrogen was investigated in gonadectomized animals in both sexes, using two different vehicles for estradiol (E2). The study in female uterus appeared to be more responsive, 6 hours after of E2 injection than ventral prostate of males. The study revealed tissue- and gene-specific changes in the acute effect of estradiol on mRNA expression. Induction of IGF-I by estradiol was reduced in PBDE 99-exposed adult offspring, both in uterus and ventral prostate. Effects of estradiol on PR (induction) and ERβ (suppression) where enhanced in uterus. This suggests to alterations in the regulation of estrogen target genes which again may be due to actions of PBDE 99 during early ontogeny or at late stages, or a combination of both. These effects are particularly difficult to discuss with PBDE 99 since its acute interaction with endocrine mechanisms is not clear. As mentioned earlier, it displays only very little, if any, estrogen agonist activity in vitro (Meerts et al., 2001) and is devoid of androgenic or anti-androgenic in vitro activity (Lichtensteiger et al., 2003). In this respect, it was

interesting to note that Gonadectomy markedly changed the mRNA expression pattern which indicates that the manifestation of the PBDE 99 effect pattern is influenced by the presence of circulating sex steroids.

We can conclude is that PBDE 99 interferes with the development of reproductive organs. These actions characterize the substance as an endocrine disruptor.

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Abbreviations

AR: Androgen Receptor

Aro10: Aroclor 1254 10mg/kg

Aro30: Aroclor 1254 30mg/kg

BDE: brominated diphenyl ethers

DP: Dorsal Prostate

ERα: Estrogen Receptor α

ERβ: Estrogen Receptor β

GD: gestational day

IGF-I: Insulin Growth Factor I

N.D.: not detectable

PR: Progesteron Receptor

PBDEs: polybrominated diphenylethers

PBDE47: 2,2',4,4'-tetrabromodiphenyl ether

PBDE99: 2,2',4,4',5-pentabromodiphenylether

PN: postnatal day

P1: PBDE 99 1mg/kg

P10: PBDE 99 10mg/kg

S.D.: standard deviation

VP: Ventral Prostate

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